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ABSTRACT

This training manual presents material on basic bacteriological laboratory procedures as required by Federal Register Water Quality Guidelines. Course topics include: characteristics, occurrences, and significance of bacterial indicators of pollution; bacteriological water quality standards and criteria; collection and handling of samples; laboratory test procedures; and data analysis. The material is designed for students who can perform basic bacteriological laboratory procedures such as simple inoculations, transfers, weighings, and related skills. Chapters include reading materials and laboratory activities. (CO)

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United States
Environmental Protection
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National Training
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Cincinnati OH 45268

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April 1980

Water

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ED209115

Bacteriological Methods in Water Quality Control Programs

Training Manual

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SE 035 924

EPA-430/1-80-004
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Bacteriological Methods in Water Quality Control Programs

This course is for laboratory personnel who can perform basic bacteriological laboratory procedures such as sample inoculations, transfers, weighings and related skills.

After successfully completing the course, the student will have increased knowledge of all aspects of sampling, analysis and data handling for bacteriological samples as required by Federal Register Guidelines for effluent monitoring and other water quality programs.

The training incorporates classroom instruction and activity sessions, student performance of laboratory assignments and follow-up discussions.

U. S. ENVIRONMENTAL PROTECTION AGENCY
Office of Water Program Operations
National Training and Operational Technology Center

FOREWORD

These manuals are prepared for reference use of students enrolled in scheduled training courses of the Office of Water Program Operations, U. S. Environmental Protection Agency.

> Due to the limited availability of the manuals it is not appropriate to cite them as technical references in bibliographies or other forms of publication.
>
> References to products and manufacturers are for illustration only; such references do not imply product endorsement by the Office of Water Program Operations, U. S. Environmental Protection Agency.

The reference outlines in this manual have been selected and developed with a goal of providing the student with a fund of the best available current information pertinent to the subject matter of the course. Individual instructors may provide additional material to cover special aspects of their own presentations.

This manual will be useful to anyone who has need for information on the subjects covered. However, it should be understood that the manual will have its greatest value as an adjunct to classroom presentations. The inherent advantages of classroom presentation is in the give-and-take discussions and exchange of information between and among students and the instructional staff.

Constructive suggestions for improvement in the coverage, content, and format of the manual are solicited and will be given full consideration.

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FEDERAL REGISTER GUIDELINES FOR CHEMICAL ANALYSES

I FEDERAL REGISTER GUIDELINES

A Authority

1 In 1972, section 304(g) of Public Law 92-500, required the EPA Administrator to promulgate guidelines establishing test procedures for the analysis of pollutants that would include the factors that must be provided in any state certification (section 401), or National Pollutant Discharge Elimination System (NPDES) permit application (section 402).

2 These test procedures are to be used by applicants to demonstrate that effluent discharges meet applicable pollutant discharge limitations, and by the states and other enforcers in routine or random monitoring of effluents to verify effectiveness of pollution control measures.

B Establishment

Following a proposed listing there was a period for reply by interested parties. The first rulemaking was published in the Federal Register on October 16, 1973.[1]

C Current Guidelines

Proposed amendments and update were published in 1975. The current guidelines were issued in the December 1, 1976[2] Federal Register.

D Format

The "Approved Test Procedures" are given in a table which lists 115 parameters, the methodology to be used to determine them and either the page number in standard references or else a source where the analytical procedure can be found.

1 Divisions

The parameters are listed alphabetically including four subcategories of related tests:

a bacteria

b metals

c radiological

d residue

2 Standard References

Those cited most often as sources of analytical procedures for the listings are the EPA Chemical Methods Manual,[3] Standard Methods,[4] ASTM[5] and U.S. Geological Survey.[6] Other sources of procedures are given in footnotes to the Table.

II EPA CHEMICAL METHODS MANUAL

A Analytical Procedures

The EPA Chemical Methods Manual was developed for their water quality laboratories, using Standard Methods and ASTM as basic references. In many cases, EPA modified methods from these sources or else developed methods suitable for their own laboratories.

B Sampling and Preservation Techniques

The manual also contains a section on sampling and preservation. This is in tabular form and contains information on volumes required for analysis, the type of container that can be used, preservation measures and holding times. The current Federal Register references this Table for recommendations on these aspects of sample handling for NPDES/Certification purposes.

C Precision and Accuracy Data

Precision and accuracy data from interlaboratory quality control studies are given for most of the methods cited.

III METHODS NOT IN 1976 GUIDELINES

A Application to Use

A system has been established for permit holders to apply for approval to use methods not listed in the December 1, 1976 Federal Register. One supplies reasons for using an alternative method to the EPA Regional Administrator through the state agency which issues certifications and/or permits. If the state does not have such an agency, the application is submitted directly to the EPA Regional Administrator.

B Order of Processing

Before approving such applications, the Regional Administrator sends a copy to the Director of the EPA Environmental Monitoring and Support Laboratory (EMSL) for review and recommendation. If the Regional Administrator rejects any application, a copy is also sent to EMSL. Within 90 days the applicant is to be notified (along with the appropriate state agency) of approval or rejection. EMSL also receives a copy of approval or rejection notifications for purposes of national coordination.

IV REQUIRED ANALYSES

Which measurements are to be done and reported depend on the specifications of the individual certifications or permits.

A Mandatory for Secondary Plants

By July 1, 1977 all municipal secondary wastewater treatment plants will be required to measure and report pH, BOD_5 (biochemical oxygen demand), suspended solids and flow. Many plants are required to report these now.

B Additional for Secondary Plants

Measurements which also may be required of secondary treatment plants are fecal coliform bacteria, residual chlorine, settleable solids, COD (chemical oxygen demand), total phosphorus, and the nitrogen series (total Kjeldahl N, NH_3-N, NO_3-N, NO_2-N).

C Municipalities and Industries

Other required analyses depend on local factors for a municipality. Each industry has requirements pertinent to the processes involved.

1 Non-specific

Non-specific measurements to assess overall water quality might be required like acidity, alkalinity, color, turbidity, specific conductance.

2 Organics

Various organic analyses might be relevant such as total organic carbon, organic nitrogen, phenols, oil and grease, surfactants, pesticides.

3 Metals

Specified metals may be of interest. Currently, the Federal Register lists 35 trace metals in the test procedure guidelines.

4 Others

Cyanide, bromide, chloride, fluoride and hardness are other measurements that might be required.

V METHODOLOGY AND SKILLS

A Methodology

The analytical methods specified in the Federal Register for these measurements range from "wet" procedures using equipment commonly found in most laboratories, to procedures requiring sophisticated instruments such as an organic carbon analyzer or an atomic absorption unit.

B Skills

The degree of analytical skills required to perform the analyses likewise varies, as does the cost of having such analyses performed by service laboratories.

VI OTHER ANALYTICAL CONSIDERATIONS

A Sample

The importance of securing a representative sample of the type (grab or composite) specified by the permit cannot be over-stressed.

B Record Keeping

Keeping complete and permanent records about the sample is also essential. Such records include conditions when the sample was collected, chain of custody signatures and details and results of analyses.

C Quality Control

Whether the analyses are done in-house or by a service laboratory, an Analytical Quality Control Program should be established. Fifteen to twenty percent of analytical time (cost) should be given to checking standard curves for colorimetry, analyzing duplicate samples to check precision and analyzing spiked samples to check accuracy. Recording precision and accuracy data on quality control charts is an effective method of using such data as a daily check on analytical performance. This can also be done with numbers reported on "blind" samples sent to service labs.

VII SUMMARY

The December 1, 1976 Federal Register promulgates guidelines establishing test procedures for the analysis of pollutants which might be required for certification (PL 92-500, section 401) or for NPDES permits (PL 92-500, section 402). The issue lists page numbers in standard references where procedures can be found to measure the 115 parameters listed. It also updates the regulations for application to use methods not cited in the guidelines. The measurements which must be made are specified by either a state agency or by U. S. EPA. Apparatus and professional skills to do the measurements will vary. Representative samples, complete records and analytical quality control measures are all necessary elements for producing reliable data.

REFERENCES

1 Federal Register, Vol 38, No 199, Tuesday, October 16, 1973, Title 40, Chapter 1, Subchapter D, Part 136, page 28758.

2 Federal Register, Vol 41, No. 232, Wednesday, December 1, 1976, Title 40, Chapter 1, Subchapter D, Part 136, page 52780.

3 Methods for Chemical Analyses of Water and Wastes, 1974, EPA, EMSL, Cincinnati, Ohio.

4 Standard Methods for the Examination of Water and Wastewater, 14th ed., 1976, APHA, Washington, D. C.

5 Annual Book of Standards, Part 31, Water, 1975, ASTM, Philadelphia, Pennsylvania.

6 Methods for Collection and Analysis of Water Samples for Dissolved Minerals and Gases, U.S.G.S. Survey Techniques of Water - Resources Inventory, Book 5, Chapter A1, 1970, U.S. GPO, Washington, D. C.

This outline was prepared by A. D. Kroner, Chemist, National Training and Operational Technology Center, MOTD, OWPO, USEPA, Cincinnati, Ohio 45268

Descriptors: Chemical analysis, chemical guidelines, self-monitoring requirements, non-approved analytical methods, NPDES federal register

WEDNESDAY, DECEMBER 1, 1976

PART II:

ENVIRONMENTAL PROTECTION AGENCY

■

WATER PROGRAMS

Guidelines Establishing Test Procedures for the Analysis of Pollutants

Amendments

Title 40—Protection of Environment

CHAPTER I—ENVIRONMENTAL PROTECTION AGENCY

SUBCHAPTER D—WATER PROGRAMS

[FRL 630-4]

PART 136—GUIDELINES ESTABLISHING TEST PROCEDURES FOR THE ANALYSIS OF POLLUTANTS

Amendment of Regulations

On June 9, 1975, proposed amendments to the Guidelines Establishing Test Procedures for the Analysis of Pollutants (40 CFR 136) were published in the FEDERAL REGISTER (40 FR 24535) as required by section 304(g) of the Federal Water Pollution Control Act Amendments of 1972 (86 Stat. 816, et seq., Pub. L. 92-500, 1972) hereinafter referred to as the Act.

Section 304(g) of the Act requires that the Administrator shall promulgate guidelines establishing test procedures for the analysis of pollutants that shall include factors which must be provided in: (1) any certification pursuant to section 401 of the Act, or (2) any permit application pursuant to section 402 of the Act. Such test procedures are to be used by permit applicants to demonstrate that effluent discharges meet applicable pollutant discharge limitations and by the States and other enforcement activities in routine or random monitoring of effluents to verify compliance with pollution control measures.

Interested persons were requested to submit written comments, suggestions, or objections to the proposed amendments by September 7, 1975. One hundred and thirty-five letters were received from commenters. The following categories of organizations were represented by the commenters: Federal agencies accounted for twenty-four responses; State agencies accounted for twenty-six responses; local agencies accounted for seventeen responses; regulated major dischargers accounted for forty-seven responses; trade and professional organizations accounted for eight responses; analytical instrument manufacturers and vendors accounted for seven responses; and analytical service laboratories accounted for six responses.

All comments were carefully evaluated by a technical review committee. Based upon the review of comments, the following principal changes to the proposed amendments were made:

(A) *Definitions.* Section 136.2 has been amended to update references: Twenty commenters, representing the entire spectrum of responding groups pointed out that the references cited in §§ 136.2(f), 136.2(g), and 136.2(h) were out-of-date; §§ 136.2(f), 136.2(g), and 136.2(h), respectively, have been amended to show the following editions of the standard references: "14th Edition of Standard Methods for the Examination of Water and Waste Water;" "1974 EPA Manual of Methods for the Analysis of Water and Waste;" and "Part 31, 1975 Annual Book of ASTM Standards."

(B) *Identification of Test Procedures.* Both the content and format of § 136.3, "Table I, List of Approved Test Procedures" have been revised in response to twenty-one comments received from State and local governments, major regulated dischargers, professional and trade associations, and analytical laboratories.

Table I has been revised by:

(1) The addition of a fourth column of references which includes procedures of the United States Geological Survey which are equivalent to previously approved methods.

(2) The addition of a fifth column of miscellaneous references to procedures which are equivalent to previously approved methods.

(3) Listing generically related parameters alphabetically within four subcategories: bacteria, metals, radiological and residue, and by listing these subcategory headings in alphabetic sequence relative to the remaining parameters.

(4) Deleting the parameter "Algicides" and by entering the single relevant algicide, "Pentachlorophenol" by its chemical name.

(C) *Clarification of Test Parameters.* The conditions for analysis of several parameters have been more specifically defined as a result of comments received by the Agency:

(1) In response to five commenters representing State or local governments, major dischargers, or analytical instrument manufacturers, the end-point for the alkalinity determination is specifically designated as pH 4.5.

(2) Manual digestion and distillation are still required as necessary preliminary steps for the Kjeldahl nitrogen procedure. Analysis after such distillation may be by Nessler color comparison, titration, electrode, or automated phenolate procedures.

(3) In response to eight commenters representative of Federal and State governments, major dischargers, and analytical instrument manufacturers, manual distillation at pH 9.5 is now specified for ammonia measurement.

(D) *New Parameters and Analytical Procedures.* Forty-four new parameters have been added to Table I. In addition to the designation of analytical procedures for these new parameters, the following modifications have been made in analytical procedures designated in response to comments.

(1) The ortho-tolidine procedure was not approved for the measurement of residual chlorine because of its poor accuracy and precision. Its approval had been requested by seven commenters representing major dischargers, State, or local governments, and analytical instrument manufacturers. Instead, the N,N-diethyl-p-phenylenediamine (DPD) method is approved as an interim procedure pending more intensive laboratory testing. It has many of the advantages of the ortho-tolidine procedure such as low cost, ease of operation, and also is of acceptable precision and accuracy.

(2) The Environmental Protection Agency concurred with the American Dye Manufacturers' request to approve its procedure for measurement of color, and copies of the procedure are now available at the Environmental Monitoring and Support Laboratory, Cincinnati (EMSL-CI).

(3) In response to three requests from Federal, State governments, and dischargers, "hardness," may be measured as the sum of calcium and magnesium analyzed by atomic absorption and expressed as their carbonates.

(4) The proposal to limit measurement of fecal coliform bacteria in the presence of chlorine to only the "Most Probable Number" (MPN) procedure has been withdrawn in response to requests from forty-five commenters including State pollution control agencies, permit holders, analysts, treatment plant operators, and a manufacturer of analytical supplies. The membrane filter (MF) procedure will continue to be an approved technique for the routine measurement of fecal coliform in the presence of chlorine. However, the MPN procedure must be used to resolve controversial situations. The technique selected by the analyst must be reported with the data.

(5) A total of fifteen objections, representing the entire spectrum of commenters, addressed the drying temperatures used for measurement of residues. The use of different temperatures in drying of total residue, dissolved residue and suspended residue was cited as not allowing direct intercomparability between these measurements. Because the intent of designating the three separate residue parameters is to measure separate waste characteristics (low drying temperatures to measure volatile substances, high drying temperatures to measure anhydrous inorganic substances), the difference in drying temperatures for these residue parameters must be preserved.

(E) *Deletion of Measurement Techniques.* Some measurement techniques that had been proposed have been deleted in response to objections raised during the public comment period.

(1) The proposed infrared spectrophotometric analysis for oil and grease has been withdrawn. Eleven commenters representing Federal or State agencies and major dischargers claimed that this parameter is defined by the measurement procedure. Any alteration in the procedure would change the definition of the parameter. The Environmental Protection Agency agreed.

(2) The proposed separate parameter for sulfide at concentrations below 1 mg/l, has been withdrawn. Methylene blue spectrophotometry is now included in Table I as an approved procedure for sulfide analysis. The titrimetric iodine procedure for sulfide analysis may only be used for analysis of sulfide at concentrations in excess of one milligram per liter.

(F) *Sample Preservation and Holding Times.* Criteria for sample preservation and sample holding times were requested by several commenters. The reference for sample preservation and holding time criteria applicable to the Table I parameters is given in footnote (1) of Table I.

(G) *Alternate Test Procedures.* Comments pertaining to § 136.4, Application for Alternate Test Procedures, included objections to various obstacles within these procedures for expeditious approval of alternate test procedures. Four analytical instrument manufacturers commented that by limiting of application for review and/or approval of alternate test procedures to NPDES permit holders, § 136.4 became an impediment to the commercial development of new or improved measurement devices based on new measurement principles. Applications for such review and/or approval will now be accepted from any person. The intent of the alternate test procedure is to allow the use of measurement systems which are known to be equivalent to the approved test procedures in waste water discharges.

Applications for approval of alternate test procedures applicable to specific discharges will continue to be made only by NPDES permit holders, and approval of such applications will be made on a case-by-case basis by the Regional Administrator in whose Region the discharge is made.

Applications for approval of alternate test procedures which are intended for nationwide use can now be submitted by any person directly to the Director of the Environmental Monitoring and Support Laboratory in Cincinnati. Such applications should include a complete methods write-up, any literature references, comparability data between the proposed alternate test procedure and those already approved by the Administrator. The application should include precision and accuracy data of the proposed alternate test procedure and data confirming the general applicability of the test procedure to the industrial categories of waste water for which it is intended. The Director of the Environmental Monitoring and Support Laboratory, after review of submitted information, will recommend approval or rejection of the application to the Administrator, or he will return the application to the applicant for more information. Approval or rejection of applications for test procedures intended for nationwide use will be made by the Administrator, after considering the recommendation made by the Director of the Environmental Monitoring and Support Laboratory, Cincinnati. Since the Agency considers these procedures for approval of alternate test procedures for nationwide use to be interim procedures, we will welcome suggestions for criteria for approval of alternate test procedures for nationwide use. Interested persons should submit their written comments in triplicate on or before June 1, 1977 to: Dr. Robert B. Medz, Environmental Protection Technologist, Monitoring Quality Assurance Standardization, Office of Monitoring and Technical Support (RD-680), Environmental Protection Agency, Washington, D.C. 20460.

(H) *Freedom of Information.* A copy of all public comments, an analysis by parameter of those comments, and documents providing further information on the rationale for the changes made in the final regulation are available for inspection and copying at the Environmental Protection Agency Public Information Reference Unit, Room 2922, Waterside Mall, 401 M Street, SW., Washington, D.C. 20460, during normal business hours. The EPA information regulation 40 CFR 2 provides that a reasonable fee may be charged for copying such documents.

Effective date: These amendments become effective on April 1, 1977.

Dated: November 19, 1976.

JOHN QUARLES,
Acting Administrator,
Environmental Protection Agency.

Chapter I, Subchapter D, of Title 40, Code of Federal Regulations is amended as follows:

1. In § 136.2, paragraphs (f), (g), and (h) are amended to read as follows:

§ 136.2 Definitions.

* * * * *

(f) "Standard Methods" means *Standard Methods for the Examination of Water and Waste Water,* 14th Edition, 1976. This publication is available from the American Public Health Association, 1015 18th Street, N.W., Washington, D.C. 20036.

(g) "ASTM" means *Annual Book of Standards, Part 31, Water, 1975.* This publication is available from the American Society for Testing and Materials, 1916 Race Street, Philadelphia, Pennsylvania 19103.

(h) "EPA Methods" means *Methods for Chemical Analysis of Water and Waste, 1974.* Methods Development and Quality Assurance Research Laboratory, National Environmental Research Center, Cincinnati, Ohio 45268; U.S. Environmental Protection Agency, Office of Technology Transfer, Industrial Environmental Research Laboratory, Cincinnati, Ohio 45268. This publication is available from the Office of Technology Transfer.

2. In § 136.3, the second sentence of paragraph (b) is amended, and a new paragraph (c) is added to read as follows:

§ 136.3 Identification of test procedures.

* * * * *

(b) * * * Under such circumstances, additional test procedures for analysis of pollutants may be specified by the Regional Administrator or the Director upon the recommendation of the Director of the Environmental Monitoring and Support Laboratory, Cincinnati.

(c) Under certain circumstances, the Administrator may approve, upon recommendation by the Director, Environmental Monitoring and Support Laboratory, Cincinnati, additional alternate test procedures for nationwide use.

3. Table I of § 136.3 is revised by listing the parameters alphabetically; by adding 44 new parameters; by adding a fourth column under references listing equivalent United States Geological Survey methods; by adding a fifth column under references listing miscellaneous equivalent methods; by deleting footnotes 1 through 7 and adding 24 new footnotes, to read as follows:

* * * * *

TABLE I.—*List of approved test procedures*[1]

Parameter and units	Method	References (page nos.) 1974 EPA methods	14th ed. standard methods	Pt. 31 1975 ASTM	USGS methods[2]	Other approved methods
1. Acidity, as $CaCO_3$, milligrams per liter.	Electrometric end point (pH of 8.2) or phenolphthalein end point.	1	273(4d)	116	40	[3](607)
2. Alkalinity, as $CaCO_3$, milligrams per liter.	Electrometric titration (only to pH 4.5) manual or automated, or equivalent automated methods.	3 5	278	111	41	[3](607)
3. Ammonia (as N), milligrams per liter.	Manual distillation[4] (at pH 9.5) followed by nesslerization, titration, electrode, Automated phenolate.	150 165 168	410 412 616	237	116	[3](614)
BACTERIA						
4. Coliform (fecal)[5], number per 100 ml.	MPN;[6] membrane filter.		922 937		[7](45)	
5. Coliform (fecal)[5] in presence of chlorine, number per 100 ml.	do.[4][6]		922 928, 937			
6. Coliform (total),[5] number per 100 ml.	do.[6]		916 928		[7](35)	
7. Coliform (total)[5] in presence of chlorine, number per 100 ml.	MPN;[6] membrane filter with enrichment.		916 933			
8. Fecal streptococci,[5] number per 100 ml.	MPN;[6] membrane filter; plate count.		943 944 947		[7](50)	
9. Benzidine, milligrams per liter.	Oxidation—colorimetric[8].					
10. Biochemical oxygen demand, 5-d (BOD_5), milligrams per liter.	Winkler (Azide modification) or electrode method.		543		[7](50)	[10](17)
11. Bromide, milligrams per liter.	Titrimetric, iodine-iodate.	14		323	58	
12. Chemical oxygen demand (COD), milligrams per liter.	Dichromate reflux.	20	550	472	124	[3](610) [10](17)
13. Chloride, milligrams per liter.	Silver nitrate; mercuric nitrate; or automated colorimetric-ferricyanide.	29 31	303 304 613	267 265	[11](46)	[3](615)

See footnotes at end of table.

Parameter and units	Method	1974 EPA methods	14th ed. standard methods	References (page nos.)		Other approved methods
				Pt. 31 1975 ASTM	USGS methods [9]	
14. Chlorinated organic compounds (except pesticides), milligrams per liter.	Gas chromatography [12]					
15. Chlorine—total residual, milligrams per liter.	Iodometric titration, amperometric or starch-iodine end-point; DPD colorimetric or Titrimetric methods (these last 2 are interim methods pending laboratory testing).	35	318 322 332 329	278		
16. Color, platinum cobalt units or dominant wave length, hue, luminance, purity.	Colorimetric; spectrophotometric; or ADMI procedure.[13]	36 39	64 66		92	
17. Cyanide, total,[14] milligrams per liter.	Distillation followed by silver nitrate titration or pyridine pyrazolone (or barbituric acid) colorimetric.	40	361	503	85	[10](22)
18. Cyanide amenable to chlorination, milligrams per liter.	do	49	376	505		
19. Dissolved oxygen, milligrams per liter.	Winkler (Azide modification) or electrode method.	51 56	443 450	368	126	[2](600)
20. Fluoride, milligrams per liter	Distillation [4] followed by ion electrode; SPADNS; or automated complexone.	65 59 61	389 391 393 614	307 306	93	
21. Hardness—Total, as $CaCO_3$, milligrams per liter.	EDTA titration; automated colorimetric; or atomic absorption (sum of Ca and Mg as their respective carbonates).	68 70	202	161	94	[2](617)
22. Hydrogen ion (pH), pH units.	Electrometric measurement	239	460	178	129	[2](606)
23. Kjeldahl nitrogen (as N), milligrams per liter.	Digestion and distillation followed by nesslerization, titration, or electrode; automated digestion automated phenolate.	175 165 182	437		122	[2](612)
METALS						
24. Aluminum—Total, milligrams per liter.	Digestion [15] followed by atomic absorption [16] or by colorimetric (Eriochrome Cyanine R).	92	152 171		[18](10)	
25. Aluminum—Dissolved, milligrams per liter.	0.45 micron filtration [17] followed by referenced methods for total aluminum.					
26. Antimony—Total, milligrams per liter.	Digestion [15] followed by atomic absorption.[16]	94				
27. Antimony—Dissolved, milligrams per liter.	0.45 micron filtration [17] followed by referenced method for total antimony.					
28. Arsenic—Total, milligrams per liter.	Digestion followed by silver diethyldithiocarbamate; or atomic absorption.[16] [18]	9 95	285 283 159		[18](31) [18](37)	
29. Arsenic—Dissolved, milligrams per liter.	0.45 micron filtration [17] followed by referenced method for total arsenic.					
30. Barium—Total, milligrams per liter.	Digestion [15] followed by atomic absorption.[16]	97	152		52	
31. Barium—Dissolved, milligrams per liter.	0.45 micron filtration [17] followed by referenced method for total barium.					
32. Beryllium—Total, milligrams per liter.	Digestion [15] followed by atomic absorption [16] or by colorimetric (Aluminon).	99	152 177		53	
33. Beryllium—Dissolved, milligrams per liter.	0.45 micron filtration [17] followed by referenced method for total beryllium.					
34. Boron—Total, milligrams per liter.	Colorimetric (Curcumin)	13	287			
35. Boron—Dissolved, milligrams per liter.	0.45 micron filtration [17] followed by referenced method for total boron.					
36. Cadmium—Total, milligrams per liter.	Digestion [15] followed by atomic absorption [16] or by colorimetric (Dithizone).	101	148 182	345	62	[2](619) [20](37)
37. Cadmium—Dissolved, milligrams per liter.	0.45 micron filtration [17] followed by referenced method for total cadmium.					
38. Calcium—Total, milligrams per liter.	Digestion [15] followed by atomic absorption; or EDTA titration.	103	148 189	346	66	
39. Calcium—Dissolved, milligrams per liter.	0.45 micron filtration [17] followed by referenced method for total calcium.					
40. Chromium VI, milligrams per liter.	Extraction and atomic absorption; colorimetric (Diphenylcarbazide).	89, 105	192		[5]78 75	
41. Chromium VI—Dissolved, milligrams per liter.	0.45 micron filtration [17] followed by referenced method for chromium VI.					
42. Chromium—Total, milligrams per liter.	Digestion [15] followed by atomic absorption [16] or by colorimetric (Diphenylcarbazide).	105	148 192	345 286	78 77	[2](619)
43. Chromium—Dissolved, milligrams per liter.	0.45 micron filtration [17] followed by referenced method for total chromium.					

See footnotes at end of table.

Parameter and units	Method	1974 EPA methods	14th ed. standard methods	References (page nos.) Pt. 31 1975 ASTM	USGS methods [9]	Other approved methods
44. Cobalt—Total, milligrams per liter.	Digestion [15] followed by atomic absorption.[16]	107	148	345	90	[10] (37)
45. Cobalt—Dissolved, milligrams per liter.	0.45 micron filtration [17] followed by referenced method for total cobalt.					
46. Copper—Total, milligrams per liter.	Digestion [15] followed by atomic absorption [16] or by colorimetric (Neocuproine).	108	148 196	345 203	83	[9] (619) [10] (37)
47. Copper—Dissolved, milligrams per liter.	0.45 micron filtration [17] followed by referenced method for total copper.					
48. Gold—Total, milligrams per liter.	Digestion [15] followed by atomic absorption.[16]					
49. Iridium—Total, milligrams per liter.	Digestion [15] followed by atomic absorption.[16]					
50. Iron—Total, milligrams per liter.	Digestion [15] followed by atomic absorption [16] or by colorimetric (Phenanthroline).	110	148 208	345 226	102	[9] (619)
51. Iron—Dissolved, milligrams per liter.	0.45 micron filtration [17] followed by referenced method for total iron.					
52. Lead—Total, milligrams per liter.	Digestion [15] followed by atomic absorption [16] or by colorimetric (Dithizone).	112	148 215	345	105	[9] (619)
53. Lead—Dissolved, milligrams per liter.	0.45 micron filtration [17] followed by referenced method for total lead.					
54. Magnesium—Total, milligrams per liter.	Digestion [15] followed by atomic absorption; or gravimetric.	114	148 221	345	109	[9] (619)
55. Magnesium—Dissolved milligrams per liter.	0.45 micron filtration [17] dollowed by referenced method for total magnesium.					
56. Manganese—Total milligrams per liter.	Digestion [15] followed by atomic absorption [16] or by colorimetric (Persulfate or periodate).	116	148 225, 227	345	111	[9] (619)
57. Manganese—Dissolved milligrams per liter.	0.45 micron filtration [17] followed by referenced method for total manganese.					
58. Mercury—Total, milligrams per liter.	Flameless atomic absorption.	118	156	338	[18] (51)	
59. Mercury—Dissolved, milligrams per liter.	0.45 micron filtration [17] followed by referenced method for total mercury.					
60. Molybdenum—Total, milligrams per liter.	Digestion [15] followed by atomic absorption.[16]	139		350		
61. Molybdenum—Dissolved, milligrams per liter.	0.45 micron filtration [17] followed by referenced method for total molybdenum.					
62. Nickel—Total, milligrams per liter.	Digestion [15] followed by atomic absorption [16] or by colorimetric (Heptoxime).	141	148	345	115	
63. Nickel—Dissolved, milligrams per liter.	0.45 micron filtration [17] followed by referenced method for total nickel.					
64. Osmium—Total, milligrams per liter.	Digestion [15] followed by atomic absorption.[16]					
65. Palladium—Total, milligrams per liter.	Digestion [15] followed by atomic absorption.[16]					
66. Platinum—Total, milligrams per liter.	Digestion [15] followed by atomic absorption.[16]					
67. Potassium—Total, milligrams per liter.	Digestion [15] followed by atomic absorption, colorimetric (Cobaltinitrite), or by flame photometric.	143	 226 234	 403	134	[9] ([illegible])
68. Potassium—Dissolved, milligrams per liter.	0.45 micron filtration [17] followed by referenced method for total potassium.					
69. Rhodium—Total, milligrams per liter.	Digestion [15] followed by atomic absorption.[16]					
70. Ruthenium—Total, milligrams per liter.	Digestion [15] followed by atomic absorption.[16]					
71. Selenium—Total, milligrams per liter.	Digestion [15] followed by atomic absorption.[16] [19]	145	159			
72. Selenium—Dissolved, milligrams per liter.	0.45 micron filtration [17] followed by referenced method for total selenium.					
73. Silica—Dissolved, milligrams per liter.	0.45 micron filtration [17] followed by colorimetric (Molybdosilicate).	274	487	398	130	
74. Silver—Total,[20] milligrams per liter.	Digestion [15] followed by atomic absorption [16] or by colorimetric (Dithizone).	146	148 243		142	[9] (619) [10] (37)
75. Silver—Dissolved,[20] milligrams per liter.	0.45 micron filtration [17] followed by referenced method for total silver.					
76. Sodium—Total, milligrams per liter.	Digestion [15] followed by atomic absorption or by flame photometric.	147	 250	 403	143	[9] ([illegible])
77. Sodium—Dissolved, milligrams per liter.	0.45 micron filtration [17] followed by referenced method for total sodium.					

See footnotes at end of table

Parameter and units	Method	1974 EPA methods	14th ed. standard methods	References (page nos.) Pt. 31 1975 ASTM	References (page nos.) USGS methods[2]	Other approved methods
78. Thallium—Total, milligrams per liter.	Digestion[15] followed by atomic absorption.[16]	149				
79. Thallium—Dissolved, milligrams per liter.	0.45 micron filtration[17] followed by referenced method for total thallium.					
80. Tin—Total, milligrams per liter.	Digestion[15] followed by atomic absorption.[16]	150			[11] (65)	
81. Tin—Dissolved, milligrams per liter.	0.45 micron filtration[17] followed by referenced method for total tin.					
82. Titanium—Total, milligrams per liter.	Digestion[15] followed by atomic absorption.[16]	151				
83. Titanium—Dissolved, milligrams per liter.	0.45 micron filtration[17] followed by referenced method for total titanium.					
84. Vanadium—Total, milligrams per liter.	Digestion[15] followed by atomic absorption[16] or by colorimetric (Gallic acid).	153	152 200	 441	 [11] (67)	
85. Vanadium—Dissolved, milligrams per liter.	0.45 micron filtration[17] followed by referenced method for total vanadium.					
86. Zinc—Total, milligrams per liter.	Digestion[15] followed by atomic absorption[16] or by colorimetric (Dithizone).	155	148 265	345	159	[3](619) [10](37)
87. Zinc—Dissolved, milligrams per liter.	0.45 micron filtration[17] followed by referenced method for total zinc.					
88. Nitrate (as N), milligrams per liter.	Cadmium reduction; brucine sulfate; automated cadmium or hydrazine reduction.[21]	201 197 207	423 427 620	 358	 119	 [3](614) [10](28)
89. Nitrite (as N), milligrams per liter.	Manual or automated colorimetric (Diazotization).	215	434		121	
90. Oil and grease, milligrams per liter.	Liquid-liquid extraction with trichloro-trifluoroethane-gravimetric.	229	515			
91. Organic carbon; total (TOC), milligrams per liter.	Combustion—Infrared method.[22]	236	532	467	[10] (4)	
92. Organic nitrogen (as N), milligrams per liter.	Kjeldahl nitrogen minus ammonia nitrogen.	175, 159	437		122	[3] (612, 614)
93. Orthophosphate (as P), milligrams per liter.	Manual or automated ascorbic acid reduction.	249 256	481 624	384	131	[3] (621)
94. Pentachlorophenol, milligrams per liter.	Gas chromatography[19]					
95. Pesticides, milligrams per liter.	do.[19]		555	529	[10] (24)	
96. Phenols, milligrams per liter.	Colorimetric (4AAP)	241	582	545		
97. Phosphorus (elemental), milligrams per liter.	Gas chromatography[20]					
98. Phosphorus; total (as P), milligrams per liter.	Persulfate digestion followed by manual or automated ascorbic acid reduction.	249 256	476, 481 624	384	133	[3] (621)
RADIOLOGICAL						
99. Alpha—Total, pCi per liter	Proportional or scintillation counter.		648	594	[10] (75+78)	
100. Alpha—Counting error, pCi per liter.	do		648	594	[10] (79)	
101. Beta—Total, pCi per liter	Proportional counter		648	601	[10] (75+78)	
102. Beta—Counting error, pCi per liter.	do		648	606	[10] (79)	
103. (a) Radium—Total, pCi per liter.	do		661	661		
(b) ^{226}Ra, pCi per liter	Scintillation counter		667		[10] (81)	
RESIDUE						
104. Total, milligrams per liter	Gravimetric, 103 to 105° C	270	91			
105. Total dissolved (filterable), milligrams per liter.	Glass fiber filtration, 180° C.	266	92			
106. Total suspended (nonfilterable), milligrams per liter.	Glass fiber filtration, 103 to 105° C.	268	94			
107. Settleable, milliliters per liter or milligrams per liter.	Volumetric or gravimetric		95			
108. Total volatile, milligrams per liter.	Gravimetric, 550° C.	272	95			
109. Specific conductance, micromhos per centimeter at 25° C.	Wheatstone bridge conductimetry.	275	71	120	148	[3] (606)
110. Sulfate (as SO_4), milligrams per liter.	Gravimetric; turbidimetric; or automated colorimetric (barium chloranilate).	 277 279	493 496	424 425		[3] (624) [3] (623)
111. Sulfide (as S), milligrams per liter.	Titrimetric—iodine for levels greater than 1 mg per liter; Methylene blue photometric.	284	505 503		154	
112. Sulfite (as SO_3), milligrams per liter.	Titrimetric, iodine-iodate	285	508	435		
113. Surfactants, milligrams per liter.	Colorimetric (Methylene blue).	157	600	494	[10] (11)	
114. Temperature, degrees C.	Calibrated glass or electrometric thermometer.	286	125		[10] (31)	
115. Turbidity, NTU	Nephelometric	295	132	223	156	

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[1] Recommendations for sampling and preservation of samples according to parameter measured may be found in "Methods for Chemical Analysis of Water and Wastes, 1974" U.S. Environmental Protection Agency, table 2, pp. viii–xii.

4. In § 136.4, the second sentence of paragraph (c) is amended by deleting the word "subchapter" immediately following the phrase "procedure under this" and immediately preceding the word "shall" and replaced with the phrase "paragraph c;" and § 136.4 is amended by adding a new paragraph (d) to read as follows:

§ 136.4 Application for alternate test procedures.

* * * * *

(c) * * * Any application for an alternate test procedure under this paragraph (c) shall: * * *

(d) An application for approval of an alternate test procedure for nationwide use may be made by letter in triplicate to the Director, Environmental Monitoring and Support Laboratory, Cincinnati, Ohio 45268. Any application for an alternate test procedure under this paragraph (d) shall:

(1) Provide the name and address of the responsible person or firm making the application.

(2) Identify the pollutant(s) or parameter(s) for which nationwide approval of an alternate testing procedure is being requested.

(3) Provide a detailed description of the proposed alternate procedure, together with references to published or other studies confirming the general applicability of the alternate test procedure to the pollutant(s) or parameter(s) in waste water discharges from representative and specified industrial or other categories.

(4) Provide comparability data for the performance of the proposed alternate test procedure compared to the performance of the approved test procedures.

§ 136.5 [Amended]

5. In § 136.5, paragraph (a) is amended by inserting the phrase "proposed by the responsible person or firm making the discharge" immediately after the words "test procedure" and before the period that ends the paragraph.

6. In § 136.5, paragraph (b) is amended by inserting in the first sentence the phrase "proposed by the responsible person or firm making the discharge" immediately after the words "such application" and immediately before the comma. The second sentence of paragraph (b) is amended by deleting the phrase "Methods Development and Quality Assurance Research Laboratory" immediately after the phrase "State Permit Program and to the Director of the" at the end of the sentence, and inserting in its place the phrase "Environmental Monitoring and Support Laboratory, Cincinnati."

7. In § 136.5, paragraph (c) is amended by inserting the phrase "proposed by the responsible person or firm making the discharge" immediately after the phrase "application for an alternate test procedure" and immediately before the comma; and by deleting the phrase "Methods Development and Quality Assurance Laboratory" immediately after the phrase "application to the Director of the" and immediately before the phrase "for review and recommendation" and inserting in its place the phrase "Environmental Monitoring and Support Laboratory, Cincinnati."

8. In § 136.5, the first sentence of paragraph (d) is amended by inserting the phrase, "proposed by the responsible person or firm making the discharge," immediately after the phrase, "application for an alternate test procedure," and immediately before the comma.

The second sentence of paragraph (d) is amended by deleting the phrase, "Methods Development and Quality Assurance Research Laboratory," immediately after the phrase, "to the Regional Administrator by the Director of the," and immediately preceding the period ending the sentence and inserting in its place the phrase, "Environmental Monitoring and Support Laboratory, Cincinnati."

The third sentence of paragraph (d) is amended by deleting the phrase, "Methods Development and Quality Assurance Research Laboratory," immediately after the phrase, "forwarded to the Director," and immediately before the second comma and by inserting in its place the phrase, "Environmental Monitoring and Support Laboratory, Cincinnati."

9. Section 136.5 is amended by the addition of a new paragraph (e) to read as follows:

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[2] All page references for USGS methods, unless otherwise noted, are to Brown, E., Skougstad, M. W., and Fishman, M. J., "Methods for Collection and Analysis of Water Samples for Dissolved Minerals and Gases," U.S. Geological Survey Techniques of Water-Resources Inv., book 5, ch. A1, (1970).

[3] EPA comparable method may be found on indicated page of "Official Methods of Analysis of the Association of Official Analytical Chemists" methods manual, 12th ed. (1975).

[4] Manual distillation is not required if comparability data on representative effluent samples are on company file to show that this preliminary distillation step is not necessary; however, manual distillation will be required to resolve any controversies.

[5] The method used must be specified.

[6] The 5 tube MPN is used.

[7] Slack, K. V. and others, "Methods for Collection and Analysis of Aquatic Biological and Mircobiological Samples: U.S. Geological Survey Techniques of Water-Resources Inv. book 5, ch. A4 (1973)."

[8] Since the membrane filter technique usually yields low and variable recovery from chlorinated wastewaters, the MPN method will be required to resolve any controversies.

[9] Adequately tested methods for benzidine are not available. Until approved methods are available, the following interim method can be used for the estimation of benzidine: (1) "Method for Benzidine and Its Salts in Wastewaters," available from Environmental Monitoring and Support Laboratory, U.S. Environmental Protection Agency, Cincinnati, Ohio 45268.

[10] American National Standard on Photographic Processing Effluents, Apr. 2, 1975. Available from ANSI, 1430 Broadway, New York, N.Y. 10018.

[11] Fishman, M. J. and Brown, Eugene, "Selected Methods of the U.S. Geological Survey for Analysis of Wastewaters," (1976) open-file report 76-177.

[12] Procedures for pentachlorophenol, chlorinated organic compounds ,and pesticides can be obtained from the Environmental Monitoring and Support Lbaoratory, U.S. Environmental Protection Agency, Cincinnati, Ohio 45268.

[13] Color method (ADMI procedure) available from Environmental Monitoring and Support Lbaoratory, U.S. Environmental Protection Agency, Cincinnati, Ohio 45268.

[14] For samples suspected of having thiocyanate interference, magnesium chloride is used as the digestion catalyst. In the approved test procedure for cyanides, the recommended catalysts are replaced with 20 ml of a solution of 510 g/l magnesium chloride ($MgCl_2 \cdot 6H_2O$). This substitution will eliminate thiocyanate interference for both total cyanide and cyanide amendable to chlorination measurements.

[15] For the determination of total metals the sample is not filtered before processing. Because vigorous digestion procedures may result in a loss of certain metals through preciptation, a less vigorous treatment is recommended as given on p. 82 (4.1.4) of "Methods for Chemical Analysis of Water and Wastes" (1974). In those instances where a more vigorous digestion is desired the procedure on p. 82 (4.1.3) should be followed. For the measurement of the noble metal series (gold, iridium, osmium, palladium, platinum, rhodium and ruthenium), an aqua regia digestion is to be substituted as follows: Transfer a representitive aliquot of the well-mixed sample to a Griffin beaker and add 3 ml of concentrated redistilled HNO_3. Place the beaker on a steam bath and evaporate to dryness. Cool the beaker and cautiously add a 5 ml portion of aqua regia. (Aqua regia is prepared immediately before use by carefully adding 3 volumes of concentrated HCl to one volume of concentrated HNO_3.) Cover the beaker with a watch glass and return to the steam bath. Continue heating the covered beaker for 50 min. Remove cover and evaporate to dryness. Cool and take up the residue in a small quantity of 1:1 HCl. Wash down the beaker walls and watch glass with distilled water and filter the sample to remove silicates and other insoluble material that could clog the atomizer. Adjust the volume to some predetermined value based on the expected metal concentration. The sample is now ready for analysis.

[16] As the various furnace devices (flameless AA) are essentially atomic absorption techniques, they are considered to be approved test methods. Methods of standard addition are to be followed as noted in p. 78 of "Methods for Chemical Analysis of Water and Wastes," 1974.

[17] Dissolved metals are defined as those constituents which will pass though a 0.45 μm membrane filter. A prefiltration is permissible to free the sample from larger suspended solids. Filter the sample as soon as practical after collection using the first 50 to 100 ml to rinse the filter flask. (Glass or plastic filtering apparatus are recommended to avoid possible contamination.) Discard the portion used to rinse the flask and collect the required volume of filtrate. Acidify the filtrate with 1:1 redistilled HNO_3 to a pH of 2. Normally, 3 ml of (1:1) acid per liter should be sufficient to preserve the samples.

[18] See "Atomic Absorption Newsletter," vol. 13, 75 (1974). Available from Perkin-Elmer Corp., Main Ave., Norwalk, Conn. 06852.

[19] Method available from Environmental Monitoring and Support Laboratory, U.S. Environmental Protection Agency, Cincinnati, Ohio 45268.

[20] Recommended methods for the analysis of silver in industrial wastewaters at concentrations of 1 mg/l and above are inadequate where silver exists as an inorganic halide. Silver halides such as the bromide and chloride are relatively insoluble in reagents such as nitric acid but are readily soluble in an aqueous buffer of sodium thiosulfate and sodium hydroxide to a pH of 12. Therefore, for levels of silver above 1 mg/l 20 ml of sample should be diluted to 100 ml by adding 40 ml each of 2M $Na_2S_2O_3$ and 2M NaOH. Standards should be prepared in the same manner. For levels of silver below 1 mg/l the recommended method is satisfactory.

[21] An automated hydrazine reduction method is available from the Environmental Monitoring and Support Laboratory, U.S. Environmental Protection Agency, Cincinnati, Ohio 45268.

[22] A number of such systems manufactured by various companies are considered to be comparable in their performance. In addition, another technique, based on combustion-methane detection is also acceptable.

[23] Goerlitz, D., Brown, E., "Methods for Analysis of Organic Substances in Water": U.S. Geological Survey Techniques of Water-Resources Inv., book 5, ch. A3 (1972).

[24] R. F. Addison and R. G. Ackman, "Direct Determination of Elemental Phosphorus by Gas-Liquid Chromatography," "Journal of Chromatography," vol. 47, No. 3, pp. 421-426, 1970.

[25] The method found on p. 75 measures only the dissolved portion while the method on p. 78 measures only suspended. Therefore, the 2 results must be added together to obtain "total."

[26] Stevens, H. H., Ficke, J. F., and Smoot, G. F., "Water Temperature—Influential Factors, Field Measurement and Data Presentation: U.S. Geological Survey Techniques of Water Resources Inv., book 1 (1975)."

§ 136.5 Approval of alternate test procedures.

* * * * *

(e) Within ninety days of the receipt by the Director of the Environmental Monitoring and Support Laboratory, Cincinnati of an application for an alternate test procedure for nationwide use, the Director of the Environmental Monitoring and Support Laboratory, Cincinnati shall notify the applicant of his recommendation to the Administrator to approve or reject the application, or shall specify additional information which is required to determine whether to approve the proposed test procedure. After such notification, an alternate method determined by the Administrator to satisfy the applicable requirements of this part shall be approved for nationwide use to satisfy the requirements of this subchapter; alternate test procedures determined by the Administrator not to meet the applicable requirements of this part shall be rejected. Notice of these determinations shall be submitted for publication in the FEDERAL REGISTER not later than 15 days after such notification and determination is made.

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BACTERIOLOGICAL INDICATORS OF WATER POLLUTION

Part 1. General Concepts

I INTRODUCTION

A Bacterial Indication of Pollution

1 In the broadest sense, a bacterial indicator of pollution is any organism which, by its presence, would demonstrate that pollution has occurred, and often suggest the source of the pollution.

2 In a more restrictive sense, bacterial indicators of pollution are associated primarily with demonstration of contamination of water, originating from excreta of warm-blooded animals (including man, domestic and wild animals, and birds).

B Implications of Pollution of Intestinal Origin

1 Intestinal wastes from warm-blooded animals regularly include a wide variety of genera and species of bacteria. Among these the coliform group may be listed, and species of the genera Streptococcus, Lactobacillus, Staphylococcus, Proteus, Pseudomonas, certain spore-forming bacteria, and others.

2 In addition, many kinds of pathogenic bacteria and other microorganisms may be released in wastes on an intermittent basis, varying with the geographic area, state of community health, nature and degree of waste treatment, and other factors. These may include the following:

a Bacteria: Species of Salmonella, Shigella, Leptospira, Brucella, Mycobacterium, and Vibrio comma.

b Viruses: A wide variety, including that of infectious hepatitis, Polioviruses, Coxsackie virus, ECHO viruses (enteric cytopathogenic human orphan -- "viruses in search of a disease"), and unspecified viruses postulated to account for outbreaks of diarrheal and upper respiratory diseases of unknown etiology, apparently infective by the water-borne route.

c Protozoa: Endamoeba histolytica

3 As routinely practiced, bacterial evidence of water pollution is a test for the presence and numbers of bacteria in wastes which, by their presence, indicate that intestinal pollution has occurred. In this context, indicator groups discussed in subsequent parts of this outline are as follows:

a Coliform group and certain subgroupings

b Fecal streptococci and certain subgroupings

c Miscellaneous indicators of water quality

4 Evidence of water contamination by intestinal wastes of warm-blooded animals is regarded as evidence of health hazard in the water being tested.

II PROPERTIES OF AN IDEAL INDICATOR OF POLLUTION

A An "ideal" bacterial indicator of pollution should:

1 Be applicable in all types of water

2 Always be present in water when pathogenic bacterial constituents of fecal contamination are present. Ramifications of this include --

  a Its density should have some direct relationship to the degree of fecal pollution.
  b It should have greater survival time in water than enteric pathogens, throughout its course of natural disappearance from the water body.
  c It should disappear rapidly from water following the disappearance of pathogens, either through natural or man-made processes.
  d It always should be absent in a bacteriologically safe water.

3 Lend itself to routine quantitative testing procedures without interference or confusion of results due to extraneous bacteria

4 Be harmless to man and other animals

B In all probability, an "ideal" bacterial indicator does not exist. The discussion of bacterial indicators of pollution in the following parts of this outline include consideration of the merits and limitations of each group, with their applications in evaluating bacterial quality of water.

III APPLICATIONS OF TESTS FOR POLLUTION INDICATORS

A Tests for Compliance with Bacterial Water Quality Standards

1 Potability tests on drinking water to meet Interstate Quarantine or other standards of regulatory agencies.

2 Determination of bacterial quality of environmental water for which quality standards may exist, such as shellfish waters, recreational waters, water resources for municipal or other supplies.

3 Tests for compliance with established standards in cases involving the protection or prosecution of municipalities, industries, etc.

B Treatment Plant Process Control

1 Water treatment plants

2 Wastewater treatment plants

C Water Quality and Pollutant Source Monitoring

1 Determination of intestinal pollution in surface water to determine type and extent of treatment required for compliance with standards

2 Tracing sources of pollution

3 Determination of effects on bacterial flora, due to addition of organic or other wastes

D Special Studies, such as

1 Tracing sources of intestinal pathogens in epidemiological investigations

2 Investigations of problems due to the Sphaerotilus group

3 Investigations of bacterial interference to certain industrial processes, with respect to such organisms as Pseudomonas, Achromobacter, or others

IV SANITARY SURVEY

The laboratory bacteriologist is not alone in evaluation of indication of water pollution of intestinal origin. On-site study (Sanitary Survey) of the aquatic environment and adjacent areas, by a qualified person, is a necessary collateral study with the laboratory work and frequently will reveal information regarding potential bacteriological hazard which may or may not be demonstrated through laboratory findings from a single sample or short series of samples.

Part 2. The Coliform Group and Its Constituents

I ORIGINS AND DEFINITION

A Background

1 In 1885, Escherich, a pioneer bacteriologist, recovered certain bacteria from human feces, which he found in such numbers and consistency as to lead him to term these organisms "the characteristic organism of human feces."

He named these organisms *Bacterium coli-commune* and *B. lactis aerogenes*. In 1895, another bacteriologist, Migula, renamed *B. coli commune* as *Escherichia coli*, which today is the official name for the type species.

2 Later work has substantiated much of the original concept of Escherich, but has shown that the above species are in fact a heterogeneous complex of bacterial species and species variants.

- a This heterogeneous group occurs not only in human feces but representatives also are to be found in many environmental media, including sewage, surface freshwaters of all categories, in and on soils, vegetation, etc.
- b The group may be subdivided into various categories on the basis of numerous biochemical and other differential tests that may be applied.

B Composition of the Coliform Group

1 Current definition

As defined in "Standard Methods for the Examination of Water and Wastewater" (14th ed): "The coliform group includes all of the aerobic and facultative anaerobic, Gram-negative, nonspore-forming rod-shaped bacteria which ferment lactose with gas formation within 48 hours at 35° C."

2 The term "coliforms" or "coliform group" is an inclusive one, including the following bacteria which may meet the definition above:

- a *Escherichia coli*, *E. aurescens*, *E. freundii*, *E. intermedia*
- b *Enterobacter aerogenes*, *E. cloacae*
- c Biochemical intermediates between the genera *Escherichia* and *Enterobacter*

3 There is no provision in the definition of coliform bacteria for "atypical" or "aberrant" coliform strains.

- a An individual strain of any of the above species may fail to meet one of the criteria of the coliform group.
- b Such an organism, by definition, is not a member of the coliform group, even though a taxonomic bacteriologist may be perfectly correct in classifying the strain in one of the above species.

II SUBDIVISION OF COLIFORMS INTO "FECAL" AND "NONFECAL" CATEGORIES

A Need

Single-test differentiations between coliforms of "fecal" origin and those of "nonfecal" origin are based on the assumption that typical *E. coli* and closely related strains are of fecal origin while *E. aerogenes* and its close relatives are not of direct fecal origin. (The latter assumption is not fully borne out by investigations at this Center. See Table 1, IMViC Type --++). A number of single differential tests have been proposed to differentiate between "fecal" and "nonfecal" coliforms.

Without discussion of their relative merits, several may be cited here:

B Types of Single-Test Differentiation

1 Determination of gas ratio

Fermentation of glucose by E. coli results in gas production, with hydrogen and carbon dioxide being produced in equal amounts. Fermentation of glucose by E. aerogenes results in generation of twice as much carbon dioxide as hydrogen.

Further studies suggested absolute correlation between H_2/CO_2 ratios and the terminal pH resulting from glucose fermentation. This led to the substitution of the methyl red test.

2 Methyl red test

Glucose fermentation by E. coli typically results in a culture medium having terminal pH in the range 4.2 - 4.6 (red color a positive test with the addition of methyl red indicator). E. aerogenes typically results in a culture medium having pH 5.6 or greater (yellow color, a negative test).

3 Indole

When tryptophane, an amino acid, is incorporated in a nutrient broth, typical E. coli strains are capable of producing indole (positive test) among the end products, whereas E. aerogenes does not (negative test).

In reviewing technical literature, the worker should be alert to the method used to detect indole formation, as the results may be greatly influenced by the analytical procedure.

4 Voges-Proskauer test (acetylmethyl carbinol test)

The test is for detection of acetylmethyl carbinol, a derivative of 2,3 butyleneglycol, as a result of glucose fermentation in the presence of peptone. E. aerogenes produces this end product (positive test) whereas E. coli gives a negative test.

a Experience with coliform cultures giving a positive test has shown a loss of this ability with storage on laboratory media for 6 months to 2½ years, in 20 - 25% of cultures (105 out of 458 cultures).

b Some workers consider that all coliform bacteria produce acetylmethyl carbinol in glucose metabolism. These workers regard acetylmethyl carbinol-negative cultures as those which have enzyme systems capable of further degradation of acetylmethyl carbinol to other end products which do not give a positive test with the analytical procedure. Cultures giving a positive test for acetylmethyl carbinol lack this enzyme system.

c This reasoning leads to a hypothesis (not experimentally proven) that the change of reaction noted in certain cultures in 4.a above is due to the activation of a latent enzyme system.

5 Citrate utilization

Cultures of E. coli are unable to use the carbon of citrates (negative test) in their metabolism, whereas cultures of E. aerogenes are capable of using the carbon of citrates in their metabolism (positive test).

Some workers (using Simmons Citrate Agar) incorporate a pH indicator (brom thymol blue) in the culture medium in order to demonstrate the typical alkaline reaction (pH 8.4 - 9.0) resulting with citrate utilization.

6 Elevated temperature (Eijkman) test

a The test is based on evidence that E. coli and other coliforms of fecal origin are capable of growing and fermenting carbohydrates (glucose or lactose) at temperatures significantly higher than the body temperature of warm-blooded animals. Organisms not associated with direct fecal origin would give a negative test result, through their inability to grow at the elevated temperature.

b While many media and techniques have been proposed, EC Broth, a medium developed by Perry and Hajna, used as a confirmatory medium for 24 hours at 44.5 ± 0.2°C are the current standard medium and method.
While the "EC" terminology of the medium suggests "E. coli" the worker should not regard this as a specific procedure for isolation of E. coli.

c A similar medium, Boric Acid Lactose Broth, has developed by Levine and his associates. This medium gives results virtually identical with those obtained from EC Broth, but requires 48 hours of incubation.

d Elevated temperature tests require incubation in a water bath. Standard Methods 14th Ed. requires this temperature to be 44.5 ± 0.2°C. Various workers have urged use of temperatures ranging between 43.0°C and 46.0°C. Most of these recommendations have provided a tolerance of ± 0.5°C from the recommended levels. However, some workers, notably in the Shellfish Program of the Public Health Service, stipulate a temperature of 44.5 ± 0.2°C. This requires use of a water bath with forced circulation to maintain this close tolerance. This tolerance range was instituted in the 13th Edition of Standard Methods and the laboratory worker should conform to these new limits.

e The reliability of elevated temperature tests is influenced by the time required for the newly-inoculated cultures to reach the designated incubation temperature. Critical workers insist on placement of the cultures in the water bath within 30 minutes, at most, after inoculation.

7 Other tests

Numerous other tests for differentiation between coliforms of fecal vs. nonfecal origin have been proposed. Current studies suggest little promise for the following tests in this application: uric acid test, cellobiose fermentation, gelatin liquefaction, production of hydrogen sulfide, sucrose fermentation, and others.

C IMViC Classification

1 In 1938, Parr reported on a review of a literature survey on biochemical tests used to differentiate between coliforms of fecal vs. nonfecal origin. A summary follows:

Test	No. of times used for differentiation
Voges-Proskauer reaction	22
Methyl red test	20
Citrate utilization	20
Indole test	15
Uric acid test	6
Cellobiose fermentation	4
Gelatin liquefaction	3
Eijkman test	2
Hydrogen sulfide production	1
Sucrose fermentation	1
a-Methyl-d-glucoside fermentation	1

2 Based on this summary and on his own studies, Parr recommended utilization of a combination of tests, the indole, methyl red, Voges-Proskauer, and the citrate utilization tests for this differentiation. This series of reactions is designated by the mnemonic "IMViC". Using this scheme, any coliform culture can be described by an "IMViC Code" according to the reactions for each culture. Thus, a typical culture of E. coli would have a code ++--, and a typical E. aerogenes culture would have a code --++.

3 Groupings of coliforms into fecal, non-fecal, and intermediate groups, as shown in "Standard Methods for the Examination of Water and Wastewater" are shown at the bottom of this page.

D Need for Study of Multiple Cultures

All the systems used for differentiation between coliforms of fecal vs. those of nonfecal origin require isolation and study of numerous pure cultures. Many workers prefer to study at least 100 cultures from any environmental source before attempting to categorize the probable source of the coliforms.

III NATURAL DISTRIBUTION OF COLIFORM BACTERIA

A Sources of Background Information

Details of the voluminous background of technical information on coliform bacteria recovered from one or more environmental media are beyond the scope of this discussion. References of this outline are suggested routes of entry for workers seeking to explore this topic.

B Studies on Coliform Distribution

1 Since 1960 numerous workers have engaged in a continuing study of the natural distribution of coliform bacteria and an evaluation of procedures for differentiation between coliforms of fecal vs. probable nonfecal origin. Results of this work have special significance because:

a Rigid uniformity of laboratory methods have been applied throughout the series of studies

b Studies are based on massive numbers of cultures, far beyond any similar studies heretofore reported

INTERPRETATION OF IMVIC REACTIONS

Organism	Indole	Methyl Red	Voges-Proskauer	Citrate
Escherichia coli	+ or -	+	-	-
Citrobacter freundii	-	+	-	+
Klebsiella-Enterobacter group	+ or -	-	+	+

c A wider variety of environmental and biological sources are being studied than in any previous series of reports.

d All studies are based on freshly recovered pure culture isolates from the designated sources.

e All studies are based on cultures recovered from the widest feasible geographic range, collected at all seasons of the year.

2 Distribution of coliform types

Table 1 shows the consolidated results of coliform distributions from various biological and environmental sources.

a The results of these studies show a high order of correlation between known or probable fecal origin and the typical E. coli IMViC code (++--). On the other hand, human feces also includes numbers of E. aerogenes and other IMViC types, which some regard as "nonfecal" segments of the coliform group. (Figure 1)

b The majority of coliforms attributable to excretal origin tend to be limited to a relatively small number of the possible IMViC codes; on the other hand, coliform bacteria recovered from undisturbed soil, vegetation, and insect life represent a wider range of IMViC codes than fecal sources, without clear dominance of any one type. (Figure 2)

c The most prominant IMViC code from nonfecal sources is the intermediate type, -+-+, which accounts for almost half the coliform cultures recovered from soils, and a high percentage of those recovered from vegetation and from insects. It would appear that if any coliform segment could be termed a "soil type" it would be IMViC code -+-+.

d It should not be surprising that cultures of typical E. coli are recovered in relatively smaller numbers from sources judged, on the basis of sanitary survey, to be unpolluted. There is no known way to exclude the influence of limited fecal pollution from small animals and birds in such environments.

e The distribution of coliform types from human sources should be regarded as a representative value for large numbers of sources. Investigations have shown that there can be large differences in the distribution of IMViC types from person to person, or even from an individual.

3 Differentiation between coliforms of fecal vs. nonfecal origin

Table 2 is a summary of findings based on a number of different criteria for differentiating between coliforms of fecal origin and those from other sources.

a IMViC type ++-- is a measurement of E. coli, Variety I, and appears to give reasonably good correlation between known or highly probable fecal origin and doubtful fecal origin.

b The combination of IMViC types, ++--, +---, and -+--, gives improved identification of probable fecal origin, and appears also to exclude most of the coliforms not found in excreta of warm-blooded animals in large numbers.

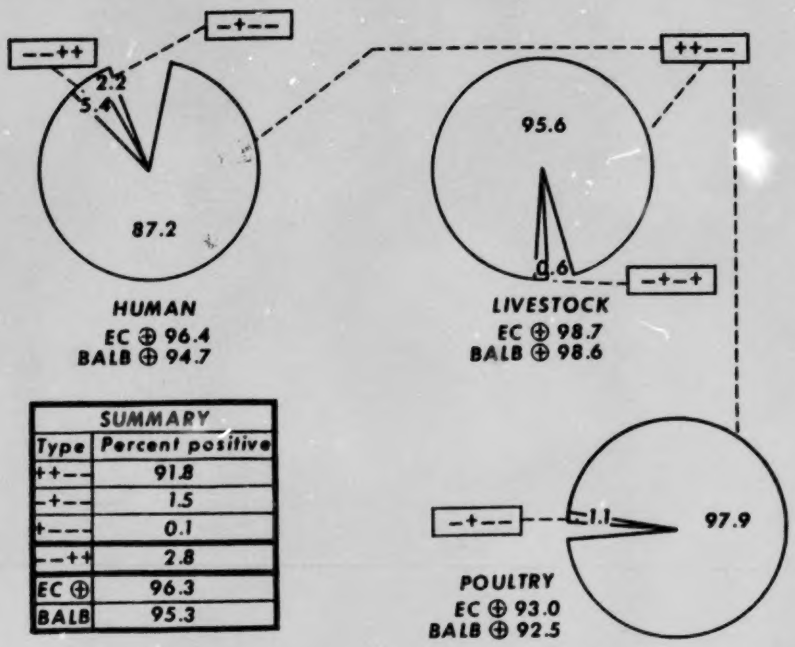
c While the indole, methyl red, Voges Proskauer, and citrate utilization tests, each used alone, appear to give useful answers when applied only to samples of known pollution from fecal sources, the interpretation is not as clear when applied to coliforms from sources believed to be remote from direct fecal pollution.

Table 1. COLIFORM DISTRIBUTION BY IMViC TYPES AND ELEVATED TEMPERATURE TEST FROM ENVIRONMENTAL AND BIOLOGICAL SOURCES

IMViC type	Vegetation		Insects		Soil				Fecal sources				Poultry	
					Undisturbed		Polluted		Human		Livestock			
	No. strains	% of total	No. strains	% of total	No. strains	% of total	No. strains	% of total	No. strains	% of total	No. strains	% of total	No. strains	% of total
++--	128	10.6	134	12.4	131	5.6	536	80.6	3932	87.2	2237	95.6	1857	97.9
--++	237	19.7	113	10.4	443	18.8	13	2.0	245	5.4	0	<0.1	1	0.1
-+--	23	1.9	0	<0.1	78	3.3	1	0.2	99	2.2	14	0.6	20	1.1
+++-	2	0.2	0	<0.1	7	0.3	0	<0.1	106	2.4	59	2.5	0	<0.1
-+-+	168	14.0	332	30.6	1131	48.1	87	13.0	50	1.1	1	<0.1	5	0.3
++-+	116	9.6	118	10.9	87	3.7	22	3.3	35	0.8	27	1.2	11	0.6
-+++	32	2.7	28	2.6	181	7.7	5	0.7	21	0.5	0	<0.1	0	<0.1
++++	291	24.2	254	23.4	159	6.8	0	<0.1	6	0.1	0	<0.1	0	<0.1
+-++	88	7.3	46	4.2	67	2.9	0	<0.1	14	0.2	0	<0.1	0	<0.1
---+	87	7.2	42	3.9	4	0.2	1	0.2	2	<0.1	0	<0.1	0	<0.1
-++-	5	0.4	0	<0.1	1	<0.1	0	<0.1	0	<0.1	0	<0.1	0	<0.1
--+-	19	1.6	0	<0.1	53	2.3	0	<0.1	0	<0.1	0	<0.1	0	<0.1
+-+-	2	0.2	0	<0.1	6	0.3	0	<0.1	0	<0.1	0	<0.1	0	<0.1
+--+	5	0.4	8	0.7	0	<0.1	0	<0.1	0	<0.1	0	<0.1	0	<0.1
+---	0	<0.1	9	0.8	0	<0.1	0	<0.1	2	<0.1	0	<0.1	2	<0.1
Total	1203		1084		2348		665		4512		2339		1896	
No. EC +	169*		162*		216		551		4349		2309		1765	
% EC +	14.1*		14.9*		9.2		82.9		96.4		98.7		93.0	

*120 of these were ++--, 15 --++, 11 -+--

*129 of these were ++--, 27 -+-+, 5 ++-+



SUMMARY	
Type	Percent positive
++––	91.8
–+––	1.5
+–––	0.1
––++	2.8
EC ⊕	96.3
BALB	95.3

FIGURE 1

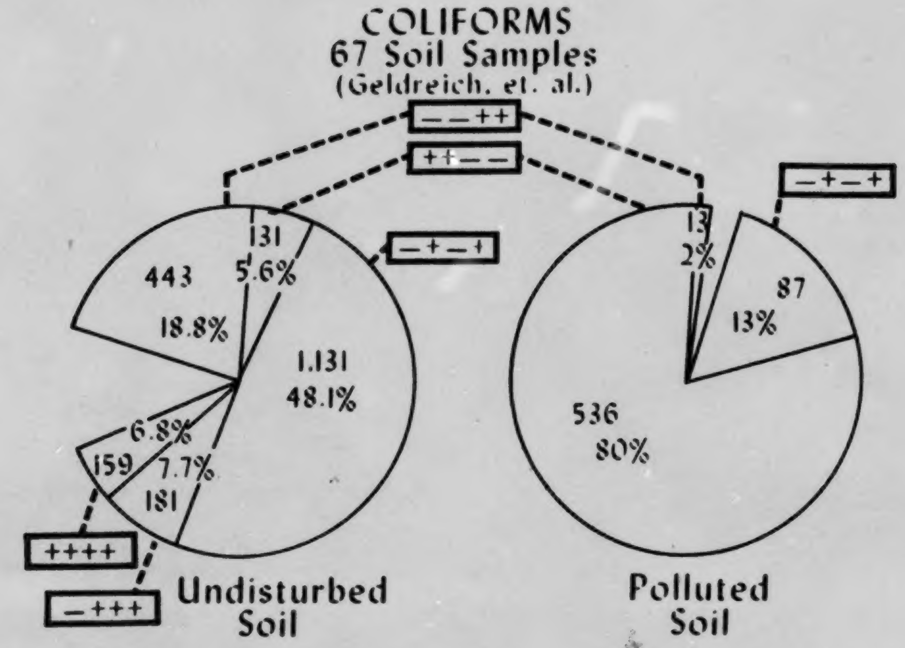

COLIFORMS
67 Soil Samples
(Geldreich, et. al.)

FIGURE 2

Table 2. COMPARISON OF COLIFORM STRAINS ISOLATED FROM WARM-BLOODED ANIMAL FECES, FROM UNPOLLUTED SOILS AND POLLUTED SOILS WITH USE OF THE IMViC REACTIONS AND THE ELEVATED TEMPERATURE TEST IN EC MEDIUM AT 44.5° C (±0.5°) (12th ed. 1965; Standard Methods for the Examination of Water and Wastewater)

Test	Warm-blooded animal feces	Soil: Unpolluted	Soil: Polluted	Vege-tation	Insects
+ + - -	91.8%	5.6%	89.6%	10.6%	12.4%
+ + - -, + - - - and - + - -	93.3%	8.9%	80.7%	12.5%	13.2%
Indole positive	94.0%	19.4%	82.7%	52.5%	52.4%
Methyl red positive	96.9%	75.6%	97.9%	63.6%	79.9%
Voges-Proskauer positive	5.1%	40.7%	97.3%	56.3%	40.6%
Citrate utilizers	3.6%	88.2%	19.2%	85.1%	86.7%
Elevated temperature (EC) positive	96.4%	9.2%	82.9%	14.1%	14.9%
Number of cultures studied	8,747	2,348	665	1,203	1,084

Total Pure Cultures Studied: 14,047

d The elevated temperature test gives excellent correlation with samples of known or highly probable fecal origin. The presence of smaller, but demonstrable, percentages of such organisms in environmental sources not interpreted as being polluted could be attributed largely to the warm-blooded wildlife in the area, including birds, rodents, and other small mammals.

e The elevated temperature test yields results equal to those obtained from the total IMViC code. It has marked advantages in speed, ease and simplicity of performance, and yields quantitative results for each water sample. Therefore, it is the official standard method for differentiation coliforms of probable direct fecal origin from those which may have become established in the bacterial flora of the aquatic or terrestrial habitat.

IV EVALUATION OF COLIFORMS AS POLLUTION INDICATORS

A The Coliform Group as a Whole

1 Merits

a The absence of coliform bacteria is evidence of a bacteriologically safe water.

b The density of coliforms is roughly proportional to the amount of excretal pollution present.

c If pathogenic bacteria of intestinal origin are present, coliform bacteria also are present, in much greater numbers.

d Coliforms are always present in the intestines of humans and other warm-blooded animals, and are eliminated in large numbers in fecal wastes.

e Coliforms are more persistent in the aquatic environment than are pathogenic bacteria of intestinal origin.

f Coliforms are generally harmless to humans and can be determined quantitatively by routine laboratory procedures.

2 Limitations

a Some of the constituents of the coliform group have a wide environmental distribution in addition to their occurrence in the intestines of warm-blooded animals.

b Some strains of the coliform group may multiply in certain polluted waters ("aftergrowth"), of high nutritive values thereby adding to the difficulty of evaluating a pollution situation in the aquatic environment. Members of the E. aerogenes section of the coliform are commonly involved in this kind of problem.

c Because of occasional aftergrowth problems, the age of the pollution may be difficult to evaluate under some circumstances.

d Tests for coliforms are subject to interferences due to other kinds of bacteria. False negative results sometimes occur when species of Pseudomonas are present. False positive results sometimes occur when two or more kinds of non-coliforms produce gas from lactose, when neither can do so alone (synergism).

B The Fecal Coliform Component of the Coliform Group (as determined by elevated temperature test)

1 Merits

a The majority (over 95% of the coliform bacteria from intestines of warm-blooded animals grow at the elevated temperature.

b These organisms are of relatively infrequent occurrence except in association with fecal pollution.

c Survival of the fecal coliform group is shorter in environmental waters than for the coliform group as whole. It follows, then, that high densities of fecal coliforms is indicative of relatively recent pollution.

d Fecal coliforms generally do not multiply outside the intestines of warm-blooded animals. In certain high-carbohydrate wastes, such as from the sugar beet refineries, exceptions have been noted.

e In some wastes, notably those from pulp and paper mills, Klebsiella has been found in large numbers utilizing the elevated temperature test. There has been much controversy about whether the occurrence of Klebsiella is due to aftergrowth due to soluble carbohydrates in such wastes. The significance of Klebsiella as an indicator of direct discharge of intestinal wastes thus is under challenge. The issue is still further complicated by questions over whether Klebsiella is in and of itself a pathogenic organism or is potentially pathogenic. This is a serious problem which is the subject of intensive research efforts.

2 Limitations

a Feces from warm-blooded animals include some (though proportionately low) numbers of coliforms which do not yield a positive fecal coliform test when the elevated temperature test is used as the criterion of differentiation. These organisms are E. coli varieties by present taxonomic classification.

b There is at present no established and consistent correlation between ratios of total coliforms/fecal coliforms in interpreting sanitary quality of environmental waters.

In domestic sewage, the fecal coliform density commonly is greater than 90% of the total coliform density. In environmental waters relatively free from recent pollution, the fecal coliform density may range from 10-30% of the total coliforms. There are, however, too many variables relating to water-borne wastes and surface water runoff to permit sweeping generalization on the numerical relationships between fecal- and total coliforms.

c Studies have been made regarding the survival of fecal coliforms in polluted waters compared with that of enteric pathogenic bacteria. In recent pollution studies, species of Salmonella have been found in the presence of 220 fecal coliforms per 100 ml (Spino), and 110 fecal coliforms per 100 ml (Brezenski, Raritan Bay Project).

3 The issue of the Klebsiella problem described in an earlier paragraph may ultimately be resolved as a merit or as a limitation of the value of the fecal coliform test.

V APPLICATIONS OF COLIFORM TESTS

A Current Status in Official Tests

1 The coliform group is designated, in "Standard Methods for the Examination of Water and Wastewater" (14th ed., 1975), through the Completed Test MPN procedure as the official test for bacteriological potability of water. The Confirmed Test MPN procedure is accepted where it has been demonstrated, through comparative tests, to yield results equivalent to the Completed Test. The membrane filter method also is accepted for examination of waters subject to interstate regulation.

2 The 12th edition of Standard Methods introduced a standard test for fecal coliform bacteria. It is emphasized that this is to be used in pollution studies, and does not apply to the evaluation of water for potability. This procedure has been continued in the 13th and 14th Editions.

B Applications

1 Tests for the coliform group as a whole are used in official tests to comply with interstate drinking water standards, state standards for shellfish waters, and in most, if not all, cases where bacterial standards of water quality have been established for such use as in recreational or bathing waters, water supplies, or industrial supplies. Laboratory personnel should be aware of possible implementation of the fecal coliform group as the official test for recreational and bathing waters.

2 The fecal coliform test has application in water quality surveys, as an adjunct to determination of total coliform density. The fecal coliform test is being used increasingly in all water quality surveys.

3 It is emphasized that no responsible worker advocates substitution of a fecal coliform test for total coliforms in evaluating drinking water quality.

Part 3. The Fecal Streptococci

I INTRODUCTION

Investigations regarding streptococci progressed from the streptococci of medical concern to those which were distributed in differing environmental conditions which, again, related to the welfare of man. The streptococci were originally reported by Laws and Andrews (1894), and Houston (1899, 1900) considered those streptococci, which we now call "fecal streptococci," as ... "indicative of dangerous pollution, since they are readily demonstrable in waters recently polluted and seemingly altogether absent from waters above suspicion of contamination.

From their discovery to the present time the fecal streptococci appear characteristic of fecal pollution, being consistently present in both the feces of all warm-blooded animals and in the environment associated with animal discharges. As early as 1910 fecal streptococci were proposed as indicators to the Metropolitan Water Board of London. However, little progress resulted in the United States until improved methods of detection and enumeration appeared after World War II.

Renewed interest in the group as indicators began with the introduction of azide dextrose broth in 1950, (Mallmann & Seligmann, 1950). The method which is in the current edition of Standard Methods appeared soon after. (Litsky, et al. 1955).

With the advent of improved methods for detection and enumeration of fecal streptococci, significant body of technical literature has appeared.

This outline will consider the findings of various investigators regarding the fecal streptococci and the significance of discharges of these organisms into the aquatic environment.

II FECAL MATERIALS

A Definition

The terms "enterococci," "fecal streptococci," "Group D streptococci," "Streptococcus fecalis," and even "streptococci" have been used in a loose and interchangeable manner to indicate the streptococci present in the enteric tract of warm-blooded animals or of the fresh fecal material excreted therefrom.

Enterococci are characterized by specific taxonomic biochemistry. Serological procedures differentiate the Group D streptococci from the various groups. Although they overlap, the three groups, fecal streptococcus, enterococcus, and Group D streptococcus, are not synonymous. Because our emphasis is on indicators of unsanitary origin, fecal streptococcus is the more appropriate term and will include the enterococcus as well as other groups.

Increasing attention is being paid to certain streptococci found in humans and certain birds whic were, at one time, considered to be biotypes of Str. faecalis or Str. faecium and therefore legitimate fecal streptococci. These are now considered to be in a separate group in their own right, the Group Q streptococci.

A rigid definition of the fecal streptococcus group is not possible with our present knowledge. The British Ministry of Health (1956) defines the organisms as "Gram-positive" cocci, generally occurring in pairs or short chains, growing in the presence of bile salt, usually capable of development at 45° C, producing acid but not gas in mannitol and lactose, failing to attack raffinose, failing to reduce nitrate to nitrite, producing acid in litmus milk and precipitating the casein in the form of a loose, but solid curd, and exhibiting a greater resistance to heat, to alkaline conditions and to high concentrations of salt than most vegetative bacteria." However, it is pointed out that "streptococci departing in one or more particulars from the type species cannot be disregarded in water."

Standard Methods (14th ed., 1975) describes the fecal streptococci as pure culture selective medium organisms which are Catalase negative and capable of originating growth in BHI broth (45°C. for 48 hours) and Bile broth medium (35° C. for 3 days).

For the proposes of this outline, and in line with the consensus of most water microbiologists in this country, the general definition of the fecal streptococci is:

> . . . "The group composed of Group D and Q species consistently present in significant numbers in fresh fecal excreta of warm-blooded animals, which includes all of the enterococcus group in addition to other groups of streptococci."

B Species Isolated

1 Findings

a Human feces

Examination of human fecal specimens yields a high percentage of the enterococcus group and usually demonstration of the S. salivarius which is generally considered a member of the human throat flora and to be surviving in human fecal materials rather than actively multiplying in the enteric tract. Also present would be a small percentage of variants or biotypes of the enterococcus group.

b Nonhuman Feces

1) Fecal material which are from nonhuman and not from fowl will yield high percentages of the S. bovis and/or S. equinus organisms with a concomitantly reduced percentage of the enterococcus group.

2) Fowl excreta

Excrement from fowl characteristically yields a large percentage of enterococcal biotypes (Group Q) as well as a significant percentage of enterococcus group.

2 Significance

Species associations with particular animal hosts is an established fact and leads to the important laboratory technique of partition counting of colonies from the membrane filter or agar pour plates in order to establish or confirm the source of excretal pollution in certain aquatic investigations.

It is important to realize that a suitable medium is necessary in order to allow all of the streptococci which we consider to be fecal streptococci to grow in order to give credence to the derived opinions. Use of liquid growth media into which direct inoculations from the sample are made have not proven to be successful for partition counting due to the differing growth rates of the various species of streptococci altering the original percentage relationships. Due to the limited survival capabilities of some of the fecal streptococci it is necessary to sample fresh fecal material or water samples in close proximity to the pollution source especially when multiple sources are contributing to a reach of water. Also the pH range must be within the range of 4.0-9.0.

Standard Methods (14th ed., 1975) now includes a schematic allowing for the identification of fecal streptococci types present within a given sample.

III FECAL STREPTOCOCCI IN THE AQUATIC ENVIRONMENT

A General

From the foregoing it is appears that the preponderant human fecal streptococci are composed of the enterococcus group and, as this is the case, several media are presently available which will detect only the enterococcal group will be suitable for use with aquatic samples which are known to be contaminated or potentially contaminated with purely domestic (human) wastes. On the other hand, when it is known or suspected that other-than-human wastes have potential egress to the aquatic environment under investigation, it is necessary to utilize those media which are capable of quantitating the whole of the fecal streptococci group.

B Stormwaters and Combined Sewers

1 General

Storm sewers are a series of pipes and conduits which receive surface runoffs from the action of rainstorms and do not include sewage which are borne by a system of sanitary sewers. Combined sewers receive both the storm runoff and the water-borne wastes of the sanitary system. Both storm water and combined sewer flows have been found to usually contain large quantities of fecal streptococci in numbers which generally are larger than those of the fecal coliform indicator organisms.

2 Bacteriological Findings

Table 1 represents, in a modified form, some of the findings of Geldreich and Kenner (1969) with respect to the densities of fecal streptococci when considering Domestic sewage in contrast to Stormwaters:

The Ratio FC/FS is that of the Fecal coliform and Fecal streptococci and it will be noted that in each case, when considering the Domestic Sewage, it is 4.0 or greater while it is less than 0.7 for stormwaters. The use of this ratio is useful to identify the source of pollution as being human or nonhuman warm-blooded animal polluted. When the ratio is greater than 4.0 it is considered to be human waste contaminated while a ratio of less than 0.7 is considered to be nonhuman. It is evident that the stormwaters have been primarily polluted by excreta of rats and other rodents and possibly domestic and/or farm animals.

Species differences are the main cause of different fecal coliform-fecal streptococci ratios. Table 2 compares fecal streptococcus and fecal coliform counts for different species. Even though individuals vary widely, masses of individuals in a species have characteristic proportion of indicators.

C Surface Waters

In general, the occurrence of fecal streptococci indicates fecal pollution and its absence indicates that little or no warm-blooded fecal contribution. In studies of remote surface waters the fecal streptococci are infrequently isolated and occurrences of small numbers can be attributed to wild life and/or snow melts and resultant drainage flows.

Various examples of fecal streptococcal occurrences are shown in Table 3 in relation to surface waters of widely varying quality. (Geldreich and Kenner 1969)

IV FECAL STREPTOCOCCI: ADVANTAGES AND LIMITATIONS

A General

Serious studies concerning the streptococci were instituted when it became apparent that they were the agents responsible or suspected for a wide variety of human diseases. Natural priority then focused itself to the taxonomy of these organisms and this study is still causing consternation as more and more microbiological techniques have been brought to bear on these questions. The sanitary microbiologist is concerned with those streptococci which inhabit the enteric tract of warm-blooded animals, their detection, and utilization in developing a criterium for water quality standards.

Table 1

DISTRIBUTION OF FECAL STREPTOCOCCI IN DOMESTIC SEWAGES AND STORMWATER RUNOFFS

Water Source	Fecal Streptococci per 100 ml median values	Ratio FC/FS
Domestic Sewage		
Preston, ID	64,000	5.3
Fargo, ND	290,000	4.5
Moorehead, MN	330,000	4.9
Cincinnati, OH	2,470,000	4.4
Lawrence, MA	4,500,000	4.0
Monroe, MI	700,000	27.9
Denver, CO	2,900,000	16.9
Stormwater		
Business District	51,000	0.26
Residential	150,000	0.04
Rural	58,000	0.05

Table 3

INDICATOR ORGANISMS IN SURFACE WATERS

Water Source	Densities/100 ml Fecal coliform	Fecal streptococci
Prairie Watersheds		
Cherry Creek, WY	90	83
Saline River, KS	95	180
Cub River, ID	110	160
Clear Creek, CO	170	110
Recreational Waters		
Lake Mead	2	444
Lake Moovalaya	9	170
Colorado River	4	256
Whitman River	32	88
Merrimack River	100	96
Public Water Intakes		
Missouri River (1959)		
Mile 470.5	11,500	39,500
Mile 434.5	22,000	79,000
Mile 408.8	14,000	59,000

Table 2. ESTIMATED PER CAPITA CONTRIBUTION OF INDICATOR MICROORGANISMS FROM SOME ANIMALS*

		Average indicator density per gram of feces		Average contribution per capita per 24 hr		
Animals	Avg wt of Feces/24 hr, wet wt, g	Fecal coliform, million	Fecal streptococci, million	Fecal coliform, million	Fecal streptococci, million	Ratio FC/FS
Man	150	13.0	3.0	2,000	450	4.4
Duck	336	33.0	54.0	11,000	18,000	0.6
Sheep	1,130	16.0	38.0	18,000	43,000	0.4
Chicken	182	1.3	3.4	240	620	0.4
Cow	23,600	0.23	1.3	5,400	31,000	0.2
Turkey	448	0.29	2.8	130	1,300	0.1
Pig	2,700	3.3	84.0	8,900	230,000	0.04

*Publication WP-20-3, P. 102

Kabler (1962) discussed the slow acceptance of the fecal streptococci as indicators of pollution resulting from:

1 Multiplicity and difficulty of laboratory procedures

2 Poor agreement between methods of quantitative enumeration

3 Lack of systematic studies of . . . .

  a sources

  b survival, and

  c interpretations, and

4 Undue attention to the S. faecalis group.

Increased attention to the fecal streptococci, especially during the last decade, have clarified many of the earlier cloudy issues and have elevated the stature of these organisms as indicators of pollution. Court precedents establishing legal status and recommendations of various technical advisory boards have placed the fecal coliform group in a position of primacy in many water quality applications. The fecal streptococci have evolved from a position of a theoretically useful indicator to one which was ancillary to the coliforms to one which was useful when discrepancies or questions evolved as to the validity of the coliform data to one where an equality status was achieved in certain applications. In the future it is anticipated that, for certain applications, the fecal streptococci will achieve a position of primacy for useful data, and, as indicated by Litsky (1955) "be taken out of the realm of stepchildren and given their legitimate place in the field of santiary bacteriology as indicators of sewage pollution."

B Advantages and Limitations

1 Survival

In general, the fecal streptococci have been observed to have a more limited survival time in the aquatic environment when compared to the coliform group. They are rivaled in this respect only by the fecal coliforms. Except for cases of persistence in waters of high electrolytic content, as may be common to irrigation waters, the fecal streptococci have not been observed to multiply in polluted waters as may sometimes be observed for some of the coliforms. Fecal streptococci usually require a greater abundance of nutrients for survival as compared to the coliforms and the coliforms are more dependent upon the oxygen tension in the waterbody. In a number of situations it was concluded that the fecal streptococci reached an extinction point more rapidly in warmer waters while the reverse was true in the colder situations as the coliforms now were totally eliminated sooner.

2 Resistance to Disinfection

In artificial pools the source of contamination by the bathers is usually limited to throat and skin flora and thus increasing attention has been paid to indicators other than those traditionally from the enteric tract. Thus, one of the organisms considered to be a fecal streptococci, namely, S. salivarius, can be a more reliable indicator when detected along with the other fecal streptococci especially since studies have confirmed the greater resistance of the fecal streptococci to chlorination. This greater resistance to chlorination, when compared to the fecal coliforms, is important since the dieoff curve differences are insignificant when the curves of the fecal coliforms are compared to various Gram negative pathogenic bacteria which reduces their effectiveness as indicators.

3 Ubiquitous Strains

Among the fecal streptococcus are two organisms, one a biotype and the other a variety of the S. faecalis, which, being ubiquitous (omnipresent) have limited sanitary significance.

The biotype, or atypical, S. faecalis is characterized by its ability to hydrolyze starch while the varietal form, liquefaciens, is nonbeta haemolytic and capable of liquefying gelatin. Quantitation of these organisms in anomalous conditions is due to their capability of survival in soil or high electrolytic waters and in waters with a temperature of less than 12 Degrees C.

Samples have been encountered which have been devoid of fecal coliforms and yet contain a substantial number of "fecal streptococci" of which these ubiquitous strains constitute the majority or all of the isolations when analyzed biochemically.

V STANDARDS AND CRITERIA

Acceptance and utilization of Total Coliform criteria, which must now be considered a pioneering effort, has largely been supplanted in concept and in fact by the fecal coliforms in establishing standards for recreational waters.

The first significant approach to the utilization of the fecal streptococci as a criterium for recreational water standards occurred in 1966 when a technical committee recommended the utilization of the fecal streptococci with the total coliforms as criteria for standards pertaining to the Calumet River and lower Lake Michigan waters. Several sets of criteria were established to fit the intended uses for this area. The use of the fecal streptococci as a criterium is indicated to be tentative pending the accumulation of existing densities and could be modified in future standards.

With the existing state-of-the-art knowledge of the presence of the fecal streptococci in waters containing low numbers of fecal coliforms it is difficult to establish a specific fecal streptococcus density limit of below 100 organisms/100 ml when used alone or in conjunction with the total coliforms.

The most useful application of the fecal streptococcus test is in the development of the fecal coliform: fecal streptococcus ratio as previously described.

Part 4. Other Bacterial Indicators of Pollution

I TOTAL BACTERIAL COUNTS

A Historical

1 The early studies of Robert Koch led him to develop tentative standards of water quality based on a limitation of not more than 100 bacterial colonies per ml on a gelatin plating medium incubated 3 days at 20° C.

2 Later developments led to inoculation of samples on duplicate plating media, with one set incubated at [illegible]° C and the other at 20° C.

- a Results were used to develop a ratio between the 37° C counts and the 20° C counts.
- b Waters having a predominant count at 37° C were regarded as being of probable sanitary significance, while those giving predominant counts at 20° C were considered to be of probable soil origin, or natural inhabitants of the water being examined.

B Groups Tested

There is no such thing as "total" bacterial count in terms of a laboratory determination.

1 Direct microscopic counts do not differentiate between living and dead cells.

2 Plate counting methods enumerate only the bacteria which are capable of using the culture medium provided, under the temperature and other growth conditions used as a standard procedure. No one culture medium and set of growth conditions can provide, simultaneously, an acceptable environment for all the heterogeneous, often conflicting, requirements of the total range of bacteria which may be recovered from waters.

C Utilization of Total Counts

1 Total bacterial counts, using plating methods, are useful for:

- a Detection of changes in the bacterial composition of a water source
- b Process control procedures in treatment plant operations
- c Determination of sanitary conditions in plant equipment or distributional systems

2 Serious limitations in total bacterial counts exist because:

- a No information is given regarding possible or probable fecal origin of bacterial changes. Large numbers of bacteria can sometimes be cultivated from waters known to be free of fecal pollution.
- b No information of any kind is given about the species of bacteria cultivated.
- c There is no differentiation between harmless or potentially dangerous forms.

3 Status of total counts

Methodology for the determination of the Standard Plate Count has been retained in the 14th Edition of Standard Methods for the stated reason:

> ..."total counts may yield useful information about the quality of water and supporting data on the significance of coliform results ... also, useful in judging the efficiency in operation of various water treatment processes and may have significant application as an in-plant control test. It is also valuable for periodic checking of finished distribution water"
> (abridged for this inclusion)

Technique for the Standard Plate Count is necessary for the performance of the Distilled Water Suitability Test as outlined in Standard Methods and elsewhere within this manual.

B Spore-Forming Bacteria (Clostridium perfringens, or C. welchii)

1 Distribution

This is one of the most widely distributed species of bacteria. It is regularly present in the intestinal tract of warm-blooded animals.

2 Nature of organism

C. perfringens is a Gram-positive, spore-forming rod. The spores cause a distinct swelling of the cell when formed. The organism is extremely active in fermentation of carbohydrates, and produces the well-known "stormy fermentation" of milk.

3 Status

The organism, when present, indicates that pollution has occurred at some time. However, because of the extremely extended viability of the spores, it is impossible to obtain even an approximation of the recency of pollution based only on the presence of C. perfringens.

The presence of the organism does not necessarily indicate an unsafe water.

C Tests for Pathogenic Bacteria of Intestinal Origin

1 Groups considered include Salmonella sp, Shigella sp, Vibrio comma, Mycobacterium sp, Pasteurella sp, Leptospira sp, and others.

2 Merits of direct tests:

Demonstration of any pathogenic species would demonstrate an unsatisfactory water quality, hazardous to persons consuming or coming into contact with that water.

3 Limitations

a There is no available routine procedure for detection of the full range of pathogenic bacteria cited above.

b Quantitative methods are not available for routine application to any of the above.

c The intermittent release of these pathogens makes it impossible to regard water as safe, even in the absence of pathogens.

d After detection, the public already would have been exposed to the organism; thus, there is no built-in margin of safety, as exists with tests for the coliform group.

4 Applications

a In tracing the source of pathogenic bacteria in epidemiological investigations

b In special research projects

c In water quality studies concerned with enforcement actions against pollution, increasing attention is being given to the demonstration of enteric pathogenic bacteria in the presence of the bacterial indicators of pollution.

D Miscellaneous Indicators

It is beyond this discussion to explore the total range of microbiological indicators of pollution that have been proposed and investigated to some extent. Mention can be made, however, of consideration of tests for the following.

1 Bacteriophages specific for any of a number of kinds of bacteria

2 Tests for Enterovirus

3 Serological procedures for detection of coliforms and other indicators: a certain amount of recent attention has been given to applications of fluorescent antibodies in such tests

4 Tests for Klebsiella

5 Tests for Pseudomonas aeruginosa

6 Tests for Salmonella

7 Tests for Fungi

8 Tests for Staphylococcus

REFERENCES

1 Standard Methods for the Examination of Water and Wastewater, 14th ed., APHA, AWWA, WPCF. Published by American Public Health Association, 1790 Broadway, New York, N.Y. 1975

2 Prescott, S.C., Winslow, C.E.A., and McCrady, M. Water Bacteriology. John Wiley & Sons, Inc. 1946.

3 Parr, L.W. Coliform Intermediates in Human Feces. Jour. Bact. 36:1. 1938.

4 Clark, H.F. and Kabler, P.W. The Physiology of the Coliform Group. Proceedings of the Rudolfs Research Conference on Principles and Applications in Aquatic Microbiology. 1963.

5 Geldreich, E.E., Bordner, R.H., Huff, C.B., Clark, H.F., and Kabler, P.W. Type Distribution of Coliform Bacteria in the Feces of Warm-Blooded Animals. JWPCF. 34:295-301. 1962.

6 Geldreich et al. The Fecal Coli-Aerogenes Flora of Soils from Various Geographic Areas. Journal of Applied Bacteriology 25:87-93. 1962.

7 Geldreich, E.E., Kenner, B.A., and Kabler, P.W. Occurrence of Coliforms, Fecal Coliforms, and Streptococci on Vegetation and Insects. Applied Microbiology. 12:63-69. 1964.

8 Kabler, P.W., Clark, H.F., and Geldreich, E.E. Sanitary Significance of Coliform and Fecal Coliform Organisms in Surface Water. Public Health Reports. 79:58-60. 1964.

9 Clark, H.F. and Kabler, P.W. Re-evaluation of the Significance of the Coliform Bacteria. Journal AWWA. 56:931-936. 1964.

10 Kenner, B.S., Clark, H.F., and Kabler, P.W. Fecal Streptococci. II. Quantification in Feces. Am. J. Public Health. 50:1553-59. 1960.

11 Litsky, W., Mallman, W.L., and Fifield, C.W. Comparison of MPN of Escherichia coli and Enterococci in River Water. Am. Jour. Public Health. 45:1949. 1955.

12 Medrek, T.F. and Litsky, W. Comparative Incidence of Coliform Bacteria and Enterococci in Undisturbed Soil. Applied Microbiology. 8:60-63. 1960.

13 Mallman, W.L., and Litsky, W. Survival of Selected Enteric Organisms in Various Types of Soil. Am. J. Public Health. 41:38-44. 1950.

14 Mallman, W.L., and Seligman, E.B., Jr. A Comparative Study of Media for Detection of Streptococci in Water and Sewage. Am. J. Public Health. 40:286-89. 1950.

15 Ministry of Health (London). The Bacterial Examination of Water Supplies. Reports on Public Health and Medical Subjects. 71:34.

16 Morris, W. and Weaver, R.H. Streptococci as Indices of Pollution in Well Water. Applied Microbiology, 2:282-285. 1954.

17 Mundt, J.O., Coggin, J.H., Jr., and Johnson, L.F. Growth of Streptococcus fecalis var. liquefaciens on Plants. Applied Microbiology. 10:552-555. 1962.

18 Geldreich, E.E. Sanitary Significance of Fecal Coliforms in the Environment. U. S. Department of the Interior. FWPCA Publ. WP-20-3. 1966.

19 Geldreich, E.E. and Kenner, B.A. Concepts of Fecal Streptococci in Stream Pollution. J. WPCF. 41:R336. 1969.

20 Kabler, P.W. Purification and Sanitary Control of Water (Potable and Waste) Ann. Rev. of Microbiol. 16:127. 1962.

21 Litsky, W., Mallman, W.L., and Fifield, C.W. Comparison of the Most Probable Numbers of Escherichia coli and Enterococci in River Waters. A.J.P.H. 45:1049. 1955.

22 Geldreich, E.E. Applying Bacteriological Parameters to Recreational Water Quality. J. AWWA. 62:113. 1970.

23 Geldreich, E.E., Best, L.C., Kenner, B.A. and Van Donsel, D.J. The Bacteriological Aspects of Stormwater Pollution. J. WPCF. 40:1860. 1968.

24 FWPCA Report of Water Quality Criteria Calumet Area - Lower Lake Michigan, Chicago, IL. Jan. 1966.

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Descriptors: Coliforms, Escherichia coli, Fecal Coliforms, Fecal Streptococci, Indicator Bacteria, Microbiology, Sewage Bacteria, Water Pollution

EXAMINATION OF WATER FOR COLIFORM AND FECAL STREPTOCOCCUS GROUPS
(Multiple Dilution Tube [MPN] Methods)

I INTRODUCTION

The subject matter of this outline is contained in three parts, as follows:

A Part 1

1 Fundamental aspects of multiple dilution tube ("most probable numbers") tests, both from a qualitative and a quantitative viewpoint.

2 Laboratory bench records.

3 Useful techniques in multiple dilution tube methods.

4 Standard supplies, equipment, and media in multiple dilution tube tests.

B Part 2

Detailed, day-by-day, procedures in tests for the coliform group and subgroups within the coliform group.

C Part 3

Detailed, day-by-day, procedures in tests for members of the fecal streptococci.

D Application of Tests to Routine Examinations

The following considerations (Table 1) apply to the selection of the Presumptive Test, the Confirmed Test, and the Completed Test. Termination of testing at the Presumptive Test level is not practiced by laboratories of this agency. It must be realized that the Presumptive Test alone has limited use when water quality is to be determined.

TABLE 1

	Examination Terminated at -		
Type of Receiving Water	Presumptive Test	Confirmed Test	Completed Test
Sewage Receiving	Applicable	Applicable	Important where results are to be used for control of raw or finished water. Application to a statistically valid number of samples from the Confirmed Test to establish its validity in determining the sanitary quality.
Treatment Plant - Raw	Applicable	Applicable	
Chlorinated	Not Done	Applicable	
Bathing	Not Done	Applicable	
Drinking	Not Done	Applicable	
Other Information		Applicable in all cases where Presumptive Test alone is unreliable.	

NOTE: Mention of commercial products and manufacturers does not imply endorsement by the Environmental Protection Agency.

II BASIS OF MULTIPLE TUBE TESTS

A Qualitative Aspects

1 For purely qualitative aspects of testing for indicator organisms, it is convenient to consider the tests applied to one sample portion, inoculated into a tube of culture medium, and the follow-up examinations and tests on results of the original inoculation. Results of testing procedures are definite: positive (presence of the organism-group is demonstrated) or negative (presence of the organism-group is not demonstrated.)

2 Test procedures are based on certain fundamental assumptions:

a First, even if only one living cell of the test organism is present in the sample, it will be able to grow when introduced into the primary inoculation medium;

b Second, growth of the test organism in the culture medium will produce a result which indicates presence of the test organism; and,

c Third, extraneous organisms will not grow, or if they do grow, they will not limit growth of the test organism; nor will they produce growth effects that will be confused with those of the bacterial group for which the test is designed.

3 Meeting these assumptions usually makes it necessary to conduct the tests in a series of stages (for example, the Presumptive, Confirmed, and Completed Test stages, respectively, of standard tests for the coliform group).

4 Features of a full, multi-stage test

a First stage: The culture medium usually serves primarily as an enrichment medium for the group tested. A good first-stage growth medium should support growth of all the living cells of the group tested, and it should include provision for indicating the presence of the test organism being studied. A first-stage medium may include some component which inhibits growth of extraneous bacteria, but this feature never should be included if it also inhibits growth of any cells of the group for which the test is designed. The Presumptive Test for the coliform group is a good example. The medium supports growth, presumably, of all living cells of the coliform group; the culture container has a fermentation vial for demonstration of gas production resulting from lactose fermentation by coliform bacteria, if present; and sodium lauryl sulfate may be included in one of the approved media for suppression of growth of certain noncoliform bacteria. This additive apparently has no adverse effect on growth of members of the coliform group in the concentration used. If the result of the first-stage test is negative, the study of the culture is terminated, and the result is recorded as a negative test. No further study is made of negative tests. If the result of the first-stage test is positive, the culture may be subjected to further study to verify the findings of the first stage.

b Second stage: A transfer is made from positive cultures of the first-stage test to a second culture medium. This test stage emphasizes provision to reduce confusion of results due to growth effects of extraneous bacteria, commonly achieved by addition of selective inhibitory agents. (The Confirmed Test for coliforms meets these requirements. Lactose and fermentation vials are provided for demonstration of coliforms in the medium. Brilliant green dye and bile salts are included as inhibitory agents which tend to suppress growth of practically all kinds of noncoliform bacteria, but do not suppress growth of coliform bacteria when used as directed).

If result of the second-stange test is negative, the study of the culture is terminated, and the result is recorded as a negative test. A negative test here means that the positive results of the first-stage test were "false positive," due to one or more kinds of extraneous bacteria. A positive second-stage test is partial confirmation of the positive results obtained in the first-stage test; the culture may be subjected to final identification through application of still further testing procedures. In routine practice, most sample examinations are terminated at the end of the second stage, on the assumption that the result would be positive if carried to the third, and final stage. This practice should be followed only if adequate testing is done to demonstrate that the assumption is valid. Some workers recommend continuing at least 5% of all sample examinations to the third stage to demonstrate the reliability of the second-stage result:

B Quantitative Aspects of Tests

1 These methods for determining bacterial numbers are based on the assumption that the bacteria can be separated from one another (by shaking or other means) resulting in a suspension of individual bacterial cells, uniformly distributed through the original sample when the primary inoculation is made.

2 Multiple dilution tube tests for quantitative determinations apply a Most Probable Number (MPN) technique. In this procedure one or more measured portions of each of a stipulated series of decreasing sample volumes is inoculated into the first-stage culture medium. Through decreasing the sample increments, eventually a volume is reached where only one cell is introduced into some tubes, and no cells are introduced into other tubes. Each of the several tubes of sample-inoculated first-stage medium is tested independently, according to the principles previously described, in the qualitative aspects of testing procedures.

3 The combination of positive and negative results is used in an application of probability mathematics to secure a single MPN value for the sample.

4 To obtain MPN values, the following conditions must be met:

a The testing procedure must result in one or more tubes in which the test organism is demonstrated to be present; and

b The testing procedure must result in one or more tubes in which the test organism is not demonstrated to be present.

5 The MPN value for a given sample is obtained through the use of MPN Tables. It is emphasized that the precision of an individual MPN value is not great when compared with most physical or chemical determinations.

6 Standard practice in water pollution surveys conducted by this organization, is to plant five tubes in each of a series of sample increments, in sample volumes decreasing at decimal intervals. For example, in testing known polluted waters, the initial sample inoculations might consist of 5 tubes each in volumes of 0.1, 0.01, 0.001, and 0.0001 ml, respectively. This series of sample volumes will yield determinate results from a low of 200 to a high of 1,600,000 organisms per 100 ml.

III LABORATORY BENCH RECORDS

A Features of a Good Bench Record Sheet

1 Provides complete identification of the sample.

2 Provides for full, day-by-day information about all tests performed on the sample.

3 Provides easy step-by-step record applicable to any portion of the sample.

4 Provides for recording of the quantitative result which will be transcribed to subsequent reports.

5 Minimizes the amount of writing by the analyst.

6 Identifies the analyst(s).

B There is no such thing as "standard" bench sheet for multiple tube tests; there are many versions of bench sheets. Some are prescribed by administrative authority (such as the Office of a State Sanitary Engineer); others are devised by laboratory or project personnel to meet specific needs.

C It is not the purpose of this discussion to recommend an "ideal" bench form; however, the form used in this training course manual is essentially similar to that used in certain research laboratories of this organization. The student enrolled in the course for which this manual is written should make himself thoroughly familiar with the bench sheet and its proper use. See Figure 1.

IV NOTES ABOUT WORKING PROCEDURES IN THE LABORATORY

A Each bacteriological examination of water by multiple dilution tube methods requires a considerable amount of manipulation; much is quite repetitious. Laboratory workers must develop and maintain good routine working habits, with constant alertness to guard against lapses into careless, slip-shod laboratory procedures and "short cuts" which only can lead to lowered quality of laboratory work.

B Specific attention is brought to the following by no means exhaustive, critical aspects of laboratory procedures in multiple dilution tube tests:

1 Original sample

a Follow prescribed care and handling procedures before testing.

b Maintain absolute identification of sample at all stages in testing.

c Vigorously shake samples (and sample dilutions) before planting in culture media.

2 Sample measurement into primary culture medium

a Sample portions must be measured accurately into the culture medium for reliable quantitative tests to be made. Standard Methods prescribes that calibration errors should not exceed ± 2.5%.

BACTERIOLOGY BENCH SHEET **Multiple Dilution Tube Tests**

Project Ohio River Survey

Sample Station Broadway Landing

Collection Data

Date 2/6/69 **Time** 8:50 **By** KJ
Temperature 8 °C **pH** 7.3
Other Observations ____________

Analytical Record

Bench Number of Sample 2
Analyst Zobel-Jeter
Test started at 11:45 **By** HLJ

ml sample	Coliform Test								Fecal coliform	Fecal Streptococcus				Remarks
	LTB		BGLB		EMB	LSTB		Gram stain		A - D		EVA		
	24	48	24	48	24	24	48		24	24	48	24	48	
10														
1.0														
0.1														
0.01														
0.001														
0.001														

Coliform MPN/100 ml
Confirmed:
Completed:
Fecal Coliform MPN:

Fecal Streptococcus MPN/100 ml
A - D - EVA:

Figure 1. SAMPLE BENCH SHEET

Suggested sample measuring practices are as follows: Mohr measuring pipets are recommended. 10 ml samples are delivered at the top of the culture tube, using 10 ml pipets. 1.0 ml samples are delivered down into the culture tube, near the surface of the medium, and "touched off" at the side of the tube when the desired amount of sample has been delivered. 1.0 ml or 2.0 ml pipets are used for measurement of this volume. 0.1 ml samples are delivered in the same manner as 1.0 ml samples, using great care that the sample actually gets into the culture medium. Only 1.0 ml pipets are used for this sample volume. After delivery of all sample increments into the culture tubes, the entire rack of culture tubes may be shaken gently to carry down any of the sample adhering to the wall of the tube above the medium.

Workers should demonstrate by actual tests that the pipets and the technique in use actually delivers the rated volumes within the prescribed limits of error.

b Volumes as small as 0.1 ml routinely can be delivered directly from the sample with suitable pipets. Lesser sample volumes first should be diluted, with subsequent delivery of suitable volumes of diluted sample into the culture medium. A diagrammatic scheme for making dilutions is shown in Figure 2.

3 Reading of culture tubes for gas production

a On removal from the incubator, shake culture rack gently, to encourage release of gas which may be supersaturated in the culture medium.

b Gas in any quantity is a positive test. It is necessary to work in conditions of suitable lighting for easy recognition of the extremely small amounts of gas inside the tops of some fermentation vials.

4 Reading of liquid culture tubes for growth as indication of a positive test requires good lighting. Growth is shown by any amount of increased turbidity or opalescence in the culture medium, with or without deposit of sediment at the bottom of the tube.

5 Transfer of cultures with inoculation loops and needles

a Always sterilize inoculation loops and needles to glowing (white hot) in flame immediately before transfer of culture; do not lay it down or touch it to any non sterile object before making the transfer.

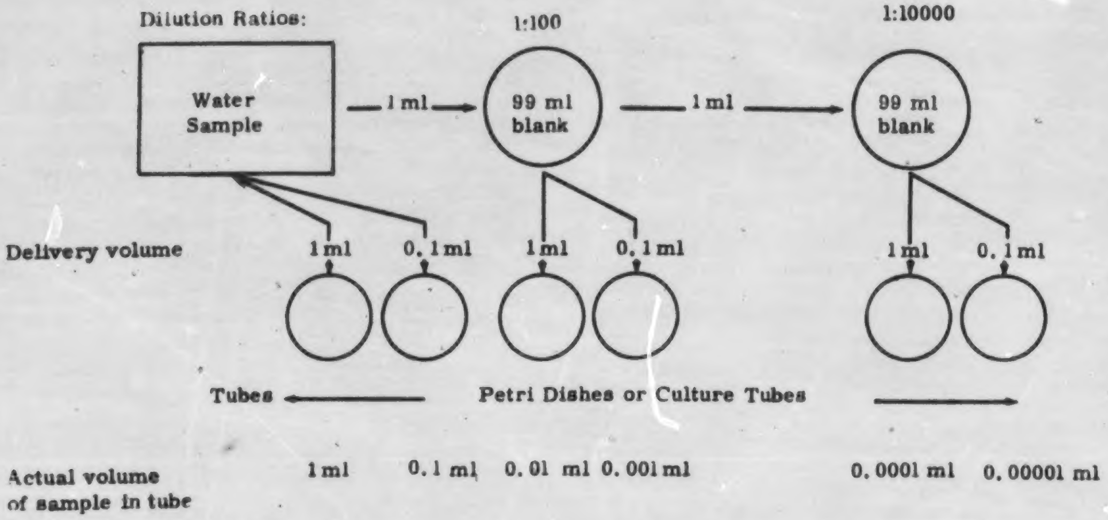
b After sterilization, allow sufficient time for cooling, in the air, to avoid heat-killing bacterial cells which will be gathered on the wire.

c Loops should be at least 3mm in inside diameter, with a capability of holding a drop of water or culture.

For routine standard transfers requiring transfer of 3 loopsful of culture, (Fecal Steptococci) many workers form three 3-mm loops on the same length of wire.

6 As an alternative to use of standard inoculation loops, the use of "applicator sticks" is described in the 14th Edition of Standard Methods.

Figure 2. PREPARATION OF DILUTIONS



The applicator sticks are dry heat sterilized (autoclave sterilization is not acceptable because of possible release of phenols if the wood is steamed) and are used on a single-service basis. Thus, for every positive culture tube transferred, a new applicator stick is used.

This use of applicator sticks is particularly attractive in field situations where it is inconvenient or impossible to provide a gas burner suitable for sterilization of the inoculation loop. In addition, use of applicator sticks is favored in laboratories where room temperatures are significantly elevated by use of gas burners.

7 Streaking cultures on agar surfaces

a All streak-inoculations should be made without breaking the surface of the agar. Learn to use a light touch with the needle; however, many inoculation needles are so sharp that they are virtually useless in this respect. When the needle is platinum or platinum-iridium wire, it sometimes is beneficial to fuse the working tip into a small sphere. This can be done by momentary insertion of a well-insulated (against electricity) wire into a carbon arc, or some other extremely hot environment. The sphere should not be more than twice the diameter of the wire from which it is formed, otherwise it will be entirely too heat-retentive to be useful.

When the needle is nichrome resistance wire, it cannot be heat-fused; the writer prefers to bend the terminal 1/16 - 1/8" of the wire at a slight angle to the overall axis of the needle. The side of the terminal bent portion of the needle then is used for inoculation of agar surfaces.

b When streaking for colony isolation, avoid using too much inoculum. The streaking pattern is somewhat variable according to individual preference. The procedure favored by the writer is shown in the accompanying figure. Note particularly that when going from any one stage of the streaking to the next, the inoculation needle is heat-sterilized.

8 Preparation of cultures for Gram stain

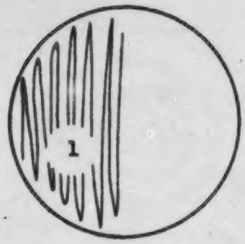
a The Gram stain always should be made from a culture grown on a nutrient agar surface (nutrient agar slants are used here) or from nutrient broth.

b The culture should be young, and should be actively growing. Many workers doubt the validity of the Gram stain made on a culture more than 24 hours old.

c Prepare a thin smear for the staining procedure. Most beginning workers tend to use too much bacterial suspension in preparing the dried smear for staining. The amount of bacteria should be so small that the dried film is barely visible to the naked eye.

V EQUIPMENT AND SUPPLIES

Consolidated lists of equipment, supplies, and culture media required for all multiple dilution tube tests described in this outline are shown in Table 2. Quantitative information is not presented; this is variable - according to the extent of the testing procedure, the number of dilutions used, and the number of replicate tubes per dilution. It is noted that requirements for alternate procedures are fully listed and choices are made in accordance to laboratory preference.

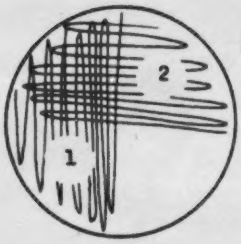


1 a Flame-sterilize an inoculation needle and air-cool.

b Dip the tip of the inoculation needle into the bacterial culture being studied.

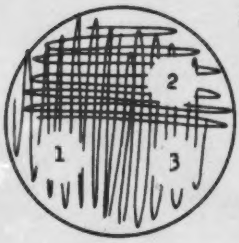
c Streak the inoculation needle tip lightly back and forth over half the agar surface, as in (1), avoiding scratching or breaking the agar surface.

d Flame-sterilize the inoculation needle and air-cool.



2 a Turn the Petri dish one-quarter-turn and streak the inoculation needle tip lightly back and forth over one-half the agar surface, working from area (1) into one-half the unstreaked area of the agar.

b Flame-sterilize the inoculation needle and air-cool.

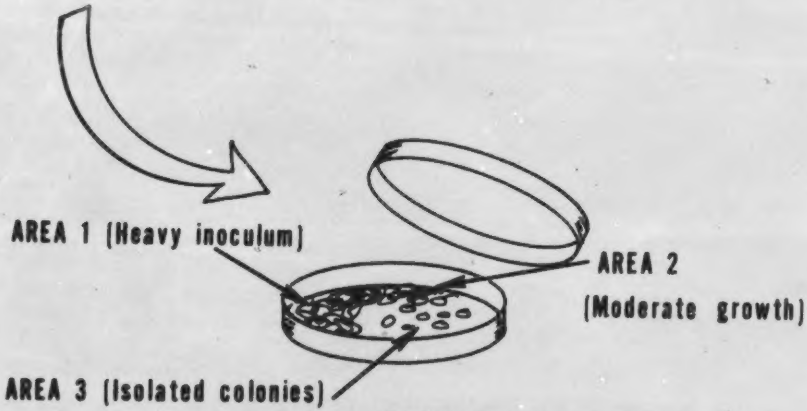


3 a Turn the Petri dish one-quarter-turn and streak the inoculation needle tip lightly back and forth over one-half the agar surface, working from area (2) into area (3), the remaining unstreaked area.

b Flame-sterilize the inoculation needle and set it aside.

c Close the culture container and incubate as prescribed.

Figure 3. A SUGGESTED PROCEDURE FOR COLONY ISOLATION BY A STREAK-PLATE TECHNIQUE



APPEARANCE OF STREAK - PLATE
AFTER INCUBATION INTERVAL

TABLE 2. APPARATUS AND SUPPLIES FOR STANDARD FERMENTATION TUBE TESTS

Description of Item	Total Coliform Group			Fecal Coliforms
	Presumptive Test	Confirmed Test	Completed Test	(EC broth)
Lauryl tryptose broth or Lactose broth. 20 ml amounts of 1.5 × concentration medium, in 25 × 150 mm culture tubes with inverted fermentation vials, suitable caps.	X			
Lauryl tryptose broth or Lactose broth, 10 ml amounts of single strength medium in 20 × 150 mm culture tubes with inverted fermentation vials, suitable caps.	X		X	
Brilliant green lactose bile broth, 2% in 10 ml amounts, single strength, in 20 × 150 mm culture tubes with inverted fermentation vials, suitable caps.		X	X	
Eosin methylene blue agar, poured in 100 × 15 mm Petri dishes		X	X	
Endo Agar, poured in 100 × 15 mm dishes		X		
Nutrient agar slant, screw cap tube			X	
EC Broth, 10 ml amounts of single strength medium in fermentation tubes.				X
Culture tube racks, 10 × 5 openings; each opening to accept 25 mm diameter tubes.	X	X	X	X
Pipettes, 10 ml, Mohr type, sterile, in suitable cans.	X			
Pipettes, 2 ml (optional), Morh type, sterile, in suitable cans	X			
Pipettes, 1 ml, Mohr type, sterile in metal suitable cans	X			
Standard buffered dilution water, sterile, 99-ml amounts in screw-capped bottles.	X			
Gas burner, Bunsen type		X	X	X
Inoculation loop, loop 3mm diameter, of nichrome or platinum-iridium wire, 26 B & S gauge, in suitable holder. (or sterile applicator stick)		X	X	X
Inoculation needle, nichrome, or platinum-iridium wire, 26 B & S gauge, in suitable holder.		X	X	
Incubator, adjusted to 35 ± 0.5°C	X	X	X	
Waterbath incubator, adjusted to 44.5 ± 0.2°C.				X
Glass microscopic slides, 1" × 3"			X	
Slide racks (optional)			X	
Gram-stain solutions, complete set			X	
Compound microscope, oil immersion lens, Abbe' condenser			X	
Basket for discarded cultures	X	X	X	X
Container for discarded pipettes	X			

Part 2

DETAILED TESTING PROCEDURES FOR MEMBERS OF THE COLIFORM GROUP BY MULTIPLE DILUTION TUBE METHODS

I SCOPE

A Tests Described

1 Presumptive Test

2 Confirmed Test

3 Completed Test

4 Fecal Coliform Test

B Form of Presentation

The Presumptive, Confirmed, and Completed Tests are presented as total, independent procedures. It is recognized that this form of presentation is somewhat repetitious, inasmuch as the Presumptive Test is preliminary to the Confirmed Test, and both the Presumptive Test and the Confirmed Test are preliminary to the Completed Test for total coliforms.

In using these procedures, the worker must know at the outset what is to be the stage at which the test is to be ended, and the details of the procedures throughout, in order to prevent the possibility of discarding gas-positive tubes before proper transfer procedures have been followed.

Thus, if the worker knows that the test will be ended at the Confirmed Test, he will turn at once to Section III, TESTING TO THE CONFIRMED TEST STAGE, and will ignore Sections II and IV.

The Fecal Coliform Test is described separately, in Section V, as an adjunct to the Confirmed Test and to the Completed Test.

II TESTING TO PRESUMPTIVE TEST STAGE

A First-Day Procedures

1 Prepare a laboratory data sheet for the sample. Record the following information: assigned laboratory number, source of sample, date and time of collection, temperature of the source, name of sample collector, date and time of receipt of sample in the laboratory. Also show the date and time of starting tests in the laboratory, name(s) of worker(s) performing the laboratory tests, and the sample volumes planted.

2 Label the tubes of lauryl tryptose broth required for the initial planting of the sample (Table 3). The label should bear three identifying marks. The upper number is the identification of the worker(s) performing the test (applicable to personnel in training courses), the number immediately below is the assigned laboratory number, corresponding with the laboratory record sheet. The lower number is the code to designate the sample volume and which tube of a replicate series is indicated.

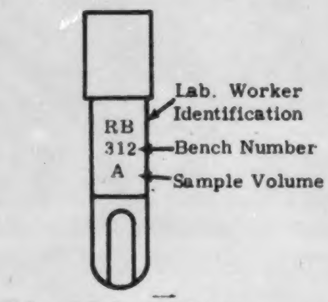
NOTE: Be sure to use tubes containing the correct concentrations of culture medium for the inoculum/tube volumes. (See the chapter on media and solutions for multiple dilution tube methods or refer to the current edition of Standard Methods for Water and Wastewater).

Table 3. SUGGESTED LABELING SCHEME FOR ORIGINAL CULTURES AND SUBCULTURES IN MULTIPLE DILUTION TUBE TESTS

	Tube 1	Tube 2	Tube 3	Tube 4	Tube 5	Sample volume represented
Bench number Volume & tube	312 A	312 B	312 C	312 D	312 E	Tubes with 10 ml of sample
Bench number Volume & tube	312 a	312 b	312 c	312 d	312 e	Tubes with 1 ml of sample
Bench number Volume & tube	312 $\underline{a}$	312 $\underline{b}$	312 $\underline{c}$	312 $\underline{d}$	312 $\underline{e}$	Tubes with 0.1 ml of sample
Bench number Volume & tube	312 1a	312 1b	312 1c	312 1d	312 1e	Tubes with 0.01 ml of sample
Bench number Volume & tube	312 2a	312 2b	312 2c	312 2d	312 2e	Tubes with 0.001 ml of sample

The labeling of cultures can be reduced by labeling only the first tube of each series of identical sample volumes in the initial planting of the sample. All subcultures from initial plantings should be labeled completely.

Typical Example



Tube of Culture Medium

3 Place the labeled culture tubes in an orderly arrangement in a culture tube rack, with the tubes intended for the largest sample volumes in the front row, and those intended for smaller volumes in the succeeding rows.

4 Shake the sample vigorously, approximately 25 times, in an arc of one foot within seven seconds and withdraw the sample portion at once.

5 Measure the predetermined sample volumes into the labeled tubes of lauryl tryptose broth, using care to avoid introduction of any bacteria into the culture medium except those in the sample.

a Use a 10 ml pipet for 10 ml sample portions, and 1 ml pipets for portions of 1 ml or less. Handle sterile pipets only near the mouthpiece, and protect the delivery end from external contamination. Do not remove the cotton plug in the mouthpiece as this is intended to protect the user from ingesting any sample.

b When using the pipet to withdraw sample portions, do not dip the pipet more than 1/2 inch into the sample; otherwise sample running down the outside of the pipet will make measurements inaccurate.

6 After measuring all portions of the sample into their respective tubes of medium, gently shake the rack of inoculated tubes to insure good mixing of sample with the culture medium. Avoid vigorous shaking, as air bubbles may be shaken into the fermentation vials and thereby invalidate the test.

7 Place the rack of inoculated tubes in the incubator at 35° ± 0.5°C for 24 ± 2 hours.

B 24-hour Procedures

1 Remove the rack of lauryl tryptose broth cultures from the incubator, and shake gently. If gas is about to appear in the fermentation vials, the shaking will speed the process.

2 Examine each tube carefully. Record, in the column "24" under LST on the laboratory data sheet, each tube showing gas in the fermentation vial as a positive (+) test and each tube not showing gas as a negative (-) test. GAS IN ANY QUANTITY IS A POSITIVE TEST.

3 Discard all gas-positive tubes of lauryl tryptose broth, and return all the gas-negative tubes to the 35°C incubator for an additional 24 ± 2 hours.

C 48-hour Procedures

1 Remove the rack of culture tubes from the incubator, read and record gas production for each tube.

2 Be sure to record all results under the 48-hour LTB column on the data sheet. Discard all tubes. The Presumptive Test is concluded at this point, and Presumptive coliforms per 100 ml can be computed according to the methods described elsewhere in this manual.

III TESTING TO CONFIRMED TEST STAGE

Note that the description starts with the sample inoculation and includes the Presumptive Test stage. The Confirmed Test preferred in Laboratories of this agency is accomplished by means of the brilliant green lactose bile broth (BGLB) and the acceptable alternate tests are mentioned in III F. In addition, the Fecal Coliform Test is included as an optional adjunct to the procedure.

A First-Day Procedures

1 Prepare a laboratory data sheet for the sample. Record the following information: assigned laboratory number, source of sample, date and time of collection, temperature of the source, name of sample collector, date and time of receipt of sample in the laboratory. Also show the date and time of starting tests in the laboratory, name(s) of worker(s) performing the laboratory tests, and the sample volumes planted.

2 Label the tubes of lauryl tryptose broth required for the initial planting of the sample. The label should bear three identifying marks. The upper number is the identification of the worker(s) performing the test (applicable to personnel in training courses), the number immediately below is the assigned laboratory number, corresponding with the laboratory record sheet. The lower number is the code to designate the sample volume and which tube of a replicate series is indicated.

NOTE: If 10-ml samples are being planted, it is necessary to use tubes containing the correct concentration of culture medium. This has previously been noted in II A-2.

3 Place the labeled culture tubes in an orderly arrangement in a culture tube rack, with the tubes intended for the largest sample volumes in the front row, and those intended for smaller volumes in the succeeding rows.

4 Shake the sample vigorously, approximately 25 times, in an up-and-down motion.

5 Measure the predetermined sample volumes into the labeled tubes of lauryl tryptose broth, using care to avoid introduction of any bacteria into the culture medium except those in the sample.

a Use a 10-ml pipet for 10 ml sample portions, and 1-ml pipets for portions of 1 ml or less. Handle sterile pipets only near the mouthpiece, and protect the delivery end from external contamination. Do not remove the cotton plug in the mouthpiece as this is intended to protect the user from ingesting any sample.

b When using the pipet to withdraw sample portions, do not dip the pipet more than 1/2 inch into the sample; otherwise sample running down the outside of the pipet will make measurements inaccurate.

c When delivering the sample into the culture medium, deliver sample portions of 1 ml or less down into the culture tube near the surface of the medium. Do not deliver small sample volumes at the top of the tube and allow them to run down inside the tube; too much of the sample will fail to reach the culture medium.

d Prepare preliminary dilutions of samples for portions of 0.01 ml or less before delivery into the culture medium. See Table 1 for preparation of dilutions. NOTE: Always deliver diluted sample portions into the culture medium as soon as possible after preparation. The interval between preparation of dilution and introduction of sample into the medium never should be as much as 30 minutes.

6 After measuring all portions of the sample into their respective tubes of medium, gently shake the rack of inoculated tubes to insure good mixing of sample with the culture medium. Avoid vigorous shaking, as air bubbles may be shaken into the fermentation vials and thereby invalidate the test.

7 Place the rack of inoculated tubes in the incubator at 35° ± 0.5°C for 24 ± 2 hours.

B 24-hour Procedures

1 Remove the rack of lauryl tryptose broth cultures from the incubator, and shake gently. If gas is about to appear in the fermentation vials, the shaking will speed the process.

2 Examine each tube carefully. Record, in the column "24" under LST on the laboratory data sheet, each tube showing gas in the fermentation vial as a positive (+) test and each tube not showing gas as a negative (-) test. GAS IN ANY QUANTITY IS A POSITIVE TEST.

3 Retain all gas-positive tubes of lauryl tryptose broth culture in their place in the rack, and proceed.

4 Select the gas-positive tubes of lauryl tryptose broth culture for Confirmed Test procedures. Confirmed Test procedures may not be required for all gas-positive cultures. If, after 24-hours of incubation, all five replicate cultures are gas-positive for two or more consecutive sample volumes, then select the set of five cultures representing the smallest volume of sample in which all tubes were gas-positive. Apply Confirmed Test procedures to all these cultures and to any other gas-positive cultures representing smaller volumes of sample, in which some tubes were gas-positive and some were gas-negative.

5 Label one tube of brilliant green lactose bile broth (BGLB) to correspond with each tube of lauryl tryptose broth selected for Confirmed Test procedures.

6 Gently shake the rack of Presumptive Test cultures. With a flame-sterilized inoculation loop transfer one loopful of culture from each gas-positive tube to the corresponding tube of BGLB. Place each newly inoculated culture into BGLB in the position of the original gas-positive tube.

7 After making the transfers, the rack should contain some 24-hour gas-negative tubes of lauryl tryptose broth and the newly inoculated BGLB.

8 If the Fecal Coliform Test is included in the testing procedures, consult Section V of this part of the outline of testing procedures.

9 Incubate the 24-hour gas-negative BGLB tubes and any newly-inoculated tubes of BGLB an additional 24 ± 2 hours at 35° ± 0.5°C.

C 48-hour Procedures

1 Remove the rack of culture tubes from the incubator, read and record gas production for each tube.

2 Some tubes will be lauryl tryptose broth and some will be brilliant green lactose bile broth (BGLB). Be sure to record results from LTB under the 48-hour LTB column and the BGLB results under the 24-hour column of the data sheet.

3 Label tubes of BGLB to correspond with all (if any) 48-hour gas-positive cultures in lauryl tryptose broth. Transfer one loopful of culture from each gas-positive LTB culture to the correspondingly-labeled tube of BGLB. NOTE: All tubes of LTB culture which were negative at 24 hours and became positive at 48 hours are to be transferred The option described above for 24-hour cultures does not apply at 48 hours.

4 If the Fecal Coliform Test is included in the testing procedure, consult Section V of the part of the outline of testing procedures.

5 Incubate the 24-hour gas-negative BGLB tubes and any newly-inoculated tubes of BGLB 24 ± 2 hours at 35° ± 0.5°C.

6 Discard all tubes of LTB and all 24-hour gas-positive BGLB cultures.

D 72-hour Procedures

1 If any cultures remain to be examined, all will be BGLB. Some may be 24 hours old and some may be 48 hours old. Remove such cultures from the incubator, examine each tube for gas production, and record results on the data sheet.

2 Be sure to record the results of 24-hour BGLB cultures in the "24" column under BGLB and the 48-hour results under the "48" column of the data sheet.

3 Return any 24-hour gas-negative cultures for incubation 24 ± 2 hours at 35 ± 0.5°C.

4 Discard all gas-positive BGLB cultures and all 48-hour gas-negative cultures from BGLB.

5 It is possible that all cultural work and results for the Confirmed Test have been finished at this point. If so, codify results and determine Confirmed Test coliforms per 100 ml as described in the outline on use of MPN Tables.

E 96-hour Procedures

At most only a few 48-hour cultures in BGLB may be present. Read and record gas production of such cultures in the "48" column under BGLB on the data sheet. Codify results and determine Confirmed Test coliforms per 100 ml.

F Streak-plate methods for the Confirmed Test, using eosin methylene blue agar or Endo agar plates, are accepted procedures in Standard Methods. The worker who prefers to use one of these media in preference to BGLB (also approved in Standard Methods) is advised to refer to the current edition of "Standard Methods for the Examination of Water and Wastewater" for procedures.

IV TESTING TO COMPLETED TEST STAGE

(Note that this description starts with the sample inoculation and proceeds through the Presumptive and the Confirmed Test stages. In addition, the Fecal Coliform Test is referred to as an optional adjunct to the procedure.)

A First-Day Procedures

1 Prepare a laboratory data sheet for the sample. Record the following information: assigned laboratory number, source of sample, date and time of collection, temperature of the source, name of sample collector, date and time of receipt of sample in the laboratory. Also show the date and time of starting tests in the laboratory, name(s) of worker(s) performing the laboratory tests, and the sample volumes planted.

2 Label the tubes of lauryl tryptose broth required for the initial planting of the sample. The label should bear three identifying marks. The upper number is the identification of the worker(s) performing the test (applicable to personnel in training courses), the number immediately below is the assigned laboratory number, corresponding with the laboratory record sheet. The lower number is the code to designate the sample volume and which tube of a replicate series is indicated. Guidance on labeling for laboratory data number and identification of individual tubes is described elsewhere in this outline.

NOTE: If 10-ml samples are being plated, it is necessary to use tubes containing the correct concentration of culture medium. This has previously been noted elsewhere in this outline and referral is made to tables.

3 Place the labeled culture tubes in an orderly arrangement in a culture tube rack, with the tubes intended for the largest sample volumes in the front row, and those intended for smaller volumes in the succeeding rows.

4 Shake the sample vigorously, approximately 25 times, in an up-and-down motion.

5 Measure the predetermined sample volumes into the labeled tubes of lauryl tryptose broth, using care to avoid introduction of any bacteria into the culture medium except those in the sample.

a Use a 10-ml pipet for 10 ml sample portions, and 1-ml pipets for portions of 1 ml or less. Handle sterile pipets only near the mouthpiece, and protect the delivery end from external contamination. Do not move the cotton plug in the mouthpiece as this is intended to protect the user from ingesting any sample.

b When using the pipet to withdraw sample portions, do not dip the pipet more than 1/2 inch into the sample; otherwise sample running down the outside of the pipet will make measurements inaccurate.

c When delivering the sample into the culture medium, deliver sample portions of 1 ml or less down into the culture tube near the surface of the medium. Do not deliver small sample volumes at the top of the tube and allow them to run down inside the tube; too much of the sample will fail to reach the culture medium.

d Prepare preliminary dilutions of samples for portions of 0.01 ml or less before delivery into the culture medium. See Table 2 for preparation of dilutions. NOTE: Always deliver diluted sample portions into the culture medium as soon as possible after preparation. The interval between preparation of dilution and introduction of sample into the medium never should be as much as 30 minutes.

6 After measuring all portions of the sample into their respective tubes of medium, gently shake the rack of inoculated tubes to insure good mixing of sample with the culture medium. Avoid vigorous shaking, as air bubbles may be shaken into the fermentation vials and thereby invalidate the test.

7 Place the rack of inoculated tubes in the incubator at 35° ± 0.5°C for 24 ± 2 hours.

B 24-hour Procedures

1 Remove the rack of lauryl tryptose broth cultures from the incubator, and shake gently. If gas is about to appear in the fermentation vials, the shaking will speed the process.

2 Examine each tube carefully. Record, in the column "24" under LST on the laboratory data sheet, each tube showing gas in the fermentation vial as a positive (+) test and each tube not showing gas as a negative (-) test. GAS IN ANY QUANTITY IS A POSITIVE TEST.

3 Retain all gas-positive tubes of lauryl tryptose broth culture in their place in the rack, and proceed.

4 Select the gas-positive tubes of lauryl tryptose broth culture for the Confirmed Test procedures. Confirmed Test procedures may not be required for all gas-positive cultures. If, after 24-hours of incubation, all five replicate cultures are gas-positive for two or more consecutive sample volumes, then select the set of five cultures representing the smallest volume of sample in which all tubes were gas-positive. Apply Confirmed Test procedures to all these cultures and to any other gas-positive cultures representing smaller volumes of sample, in which some tubes were gas-positive and some were gas-negative.

5 Label one tube of brilliant green lactose bile broth (BGLB) to correspond with each tube of lauryl tryptose broth selected for Confirmed Test procedures.

6 Gently shake the rack of Presumptive Test cultures. With a flame-sterilized inoculation loop transfer one loopful of culture from each gas-positive tube to the corresponding tube of BGLB. Place each newly inoculated culture into BGLB in the position of the original gas-positive tube.

7 If the Fecal Coliform Test is included in the testing procedure, consult Section V of this outline for details of the testing procedure.

8 After making the transfer, the rack should contain some 24-hour gas-negative tubes of lauryl tryptose borth and the newly inoculated BGLB. Incubate the rack of cultures at 35°C ± 0.5°C for 24 ± 2 hours.

C 48-hour Procedures

1 Remove the rack of culture tubes from the incubator, read and record gas production for each tube.

2 Some tubes will be lauryl tryptose broth and some will be brilliant green lactose

bile broth (BGLB). Be sure to record results from LTB under the 48-hour LTB column and the BGLB results under the 24-hour column of the data sheet.

3 Label tubes of BGLB to correspond with all (if any) 48-hour gas-positive cultures in lauryl tryptose broth. Transfer one loopful of culture from each gas-positive LTB culture to the correspondingly-labeled tube of BGLB. NOTE: All tubes of LTB culture which were negative at 24 hours and became positive at 48 hours are to be transferred. The Option described above for 24-hour LTB cultures does not apply at 48 hours.

4 Incubate the 24-hour gas-negative BGLB tubes and any newly-inoculated tubes of BGLB 24 ± 2 hours at 35° ± 0.5°C. Retain all 24-hour gas-positive cultures in BGLB for further test procedures.

5 Label a Petri dish preparation of eosin methylene blue agar (EMB agar) to correspond with each gas-positive culture in BGLB.

6 Prepare a streak plate for colony isolation from each gas-positive culture in BGLB on the correspondingly-labeled EMB agar plate.

Incubate the EMB agar plates 24 ± 2 hours at 35° ± 0.5°C.

D 72-hour Procedures

1 Remove the cultures from the incubator. Some may be on BGLB; several EMB agar plates also can be expected.

2 Examine and record gas production results for any cultures in BGLB.

3 Retain any gas-positive BGLB cultures and prepare streak plate inoculations for colony isolation in EMB agar. Incubate the EMB agar plates 24 ± 2 hours at 35 ± 0.5° C. Discard the gas-positive BGLB cultures after transfer.

4 Reincubate any gas-negative BGLB cultures 24 ± 2 hours at 35° ± 0.5°C.

5 Discard all 48-hour gas-negative BGLB cultures.

6 Examine the EMB agar plates for the type of colonies developed thereon. Well-isolated colonies having a dark center (when viewed from the lower side, held toward a light) are termed "nucleated or fisheye" colonies, and are regarded as "typical" coliform colonies. A surface sheen may or may not be present on "typical" colonies. Colonies which are pink or opaque but are not nucleated are regarded as "atypical colonies." Other colony types are considered "noncoliform." Read and record results as + for "typical" (nucleated) colonies ± for "atypical" (non-nucleated pink or opaque colonies), and - for other types of colonies which might develop.

7 With plates bearing "typical" colonies, select at least one well-isolated colony and transfer it to a correspondingly-labeled tube of lactose broth and to an agar slant. As a second choice, select at least two "atypical" colonies (if typical colonies are not present) and transfer them to labeled tubes of lactose broth and to agar slants. As a third choice, in the absence of typical or atypical coliform-like colonies, select at least two well-isolated colonies representative of those appearing on the EMB plate, and transfer them to lactose broth and to agar slants.

8 Incubate all cultures transfered from EMB agar plates 24 ± 2 hours at 35 ± 0.5°C.

E 96-hour Procedures

1 Subcultures from the samples being studied may include: 48-hour tubes of BGLB, EMB agar plates, lactose broth tubes, and agar slant cultures.

2 If any 48-hour tubes of BGLB are present, read and record gas production in the "48" column under BGLB. From any gas-positive BGLB cultures prepare streak plate inoculations for colony isolation on EMB agar. Discard all tubes of BGLB, and incubate EMB agar plates 24 ± 2 hours at 35 ± 0.5°C.

3 If any EMB plates are present, examine and record results in the "EMB" column of the data sheet. Make transfers to agar slants and to lactose broth from all EMB agar plate cultures. In decreasing order of preference, transfer at least one typical colony, or at least two atypical colonies, or at least two colonies representative of those on the plate.

4 Examine and record results from the lactose broth cultures.

5 Prepare a Gram-stained smear from each of the agar slant cultures, as follows:

NOTE: Always prepare Gram stain from an actively growing culture, preferably about 18 hours old, and never more than 24 hours old. Failure to observe this precaution often results in irregular staining reactions.

- a Thoroughly clean a glass slide to free it of any trace of oily film.
- b Place one drop of distilled water on the slide.
- c Use the inoculation needle to suspend a tiny amount of growth from the nutrient agar slant culture in the drop of water.
- d Mix the thin suspension of cells with the tip of the inoculation needle, and allow the water to evaporate.
- e "Fix" the smear by gently warming the slide over a flame.
- f Stain the smear by flooding it for 1 minute with ammonium oxylate-crystal violet solution.
- g Flush the excess dye solution off in gently running water.
- h Flood the smear with Lugol's iodine for 1 minute.
- i Wash the slide in gently running water.
- j Decolorize the smear with acetone alcohol solution with gentle agitation for 10-30 seconds, depending upon extent of removal of crystal violet dye.
- k Counterstain for 10 seconds with safranin solution, then wash in running water and gently blot dry with bibulous paper.
- l Examine the slide under the microscope, using the oil immersion lens. Coliform bacteria are Gram-negative (pink to red color and nonspore-forming, rod-shaped cells, occurring singly, in pairs, or rarely in short chains.
- m If typical coliform staining reaction and morphology are observed, record + in in the appropriate space under the "Gram Stain" column of the data sheet. If typical morphology and staining reaction are not observed, then mark it + or -, and make suitable comment in the "remarks" column at the right-hand side of the data sheet.
- n If spore-forming bacteria are observed, it will be necessary to repurify the culture from which the observations were made. Consult the instructor, or refer to Standard Methods, for procedures.

At this point, it is possible that all cultural work for the Completed Test has been finished. If so, codify results and determine Completed Test coliforms per 100 ml.

F 120-hour Procedures and following:

1 Any procedures to be undertaken from this point are "straggler" cultures on media already described, and requiring step-by-step methodology already given in detail. Such cultures may be on: EMB plates, agar slants, or lactose broth. The same time-and-temperature of incubation required for earlier studies applies to the "stragglers" as do the observations, staining reactions, and interpretation of results. On conclusion of all cultural procedures, codify results and determine Completed Test coliforms per 100 ml.

V FECAL COLIFORM TEST

A General Information

1 The procedure described is an elevated temperature test for fecal coliform bacteria.

2 Equipment required for the tests are those required for the Presumptive Test of Standard Methods, a water-bath incubator, and the appropriate culture media.

B Fecal Coliform Test with EC Broth

1 Sample: The test is applied to gas-positive tubes from the Standard Methods Presumptive Test (lauryl tryptose broth), in parallel with Confirmed Test procedures.

2 24-hour Operations. Initial procedures are the planting procedures described for the Standard Methods Presumptive Coliform test.

a After reading and recording gas-production on lauryl tryptose broth, temporarily retain all gas-positive tubes.

b Label a tube of EC broth to correspond with each gas-positive tube of lauryl tryptose broth. The option regarding transfer of only a limited number of tubes to the Confirmed Test sometimes can be applied here. However, the worker is urged to avoid exercise of this option until he has assured the applicability of the option by preliminary tests on the sample source.

c Transfer one loopful of culture from each gas-positive culture in lauryl tryptose broth to the correspondingly labeled tube of EC broth.

d Incubate EC broth tubes 24 ± 2 hours at 44.5 ± 0.2°C in a waterbath with water depth sufficient to come up at least as high as the top of the culture medium in the tubes. Place in waterbath as soon as possible after inoculation and always within 30 minutes after inoculation.

3 48-hour operations

a Remove the rack of EC cultures from the waterbath, shake gently, and record gas production for each tube. Gas in any quantity is a positive test.

b As soon as results are recorded, discard all tubes. (This is a 24-hour test for EC broth inoculations and not a 48-hour test.)

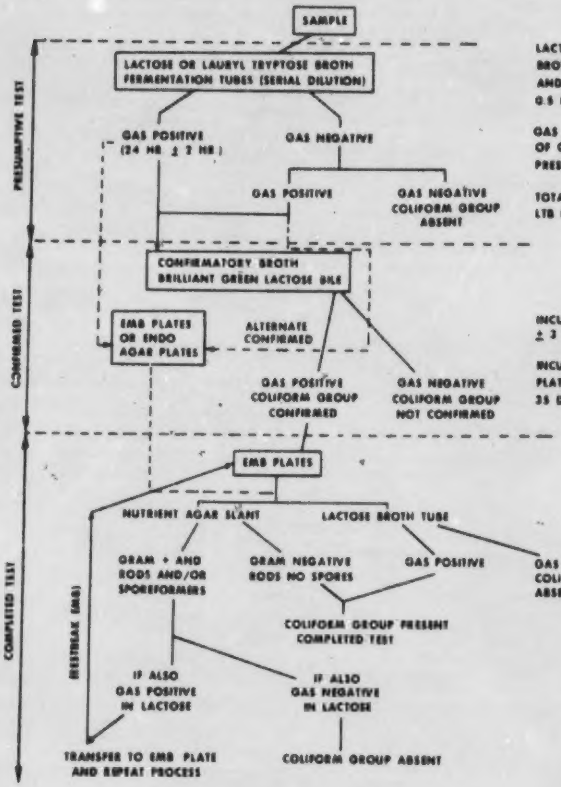
c Transfer any additional 48-hour gas-positive tubes of lauryl tryptose broth to correspondingly labeled tubes of EC broth. Incubate 24 ± 2 hours at 44.5 ± 0.2°C.

4 72-hour operations

a Read and record gas production for each tube. Discard all cultures.

b Codify results and determine fecal coliform count per 100 ml of sample.

TESTS FOR COLIFORM GROUP


SAMPLE
LACTOSE OR LAURYL TRYPTOSE BROTH FERMENTATION TUBES (SERIAL DILUTION)
PRESUMPTIVE TEST
GAS POSITIVE (24 HR. ± 2 HR.)
GAS NEGATIVE
GAS POSITIVE
GAS NEGATIVE COLIFORM GROUP ABSENT
CONFIRMED TEST
CONFIRMATORY BROTH BRILLIANT GREEN LACTOSE BILE
EMB PLATES OR ENDO AGAR PLATES
ALTERNATE CONFIRMED
GAS POSITIVE COLIFORM GROUP CONFIRMED
GAS NEGATIVE COLIFORM GROUP NOT CONFIRMED
COMPLETED TEST
EMB PLATES
NUTRIENT AGAR SLANT
LACTOSE BROTH TUBE
RESTREAK (EMB)
GRAM + AND RODS AND/OR SPOREFORMERS
GRAM NEGATIVE RODS NO SPORES
GAS POSITIVE
GAS NEGATIVE COLIFORM GROUP ABSENT
COLIFORM GROUP PRESENT COMPLETED TEST
IF ALSO GAS POSITIVE IN LACTOSE
IF ALSO GAS NEGATIVE IN LACTOSE
TRANSFER TO EMB PLATE AND REPEAT PROCESS
COLIFORM GROUP ABSENT

LACTOSE & LAURYL TRYPTOSE BROTH ARE INTERCHANGEABLE MEDIA AND ARE INCUBATED AT 35 DEG C± 0.5 DEG C.

GAS POSITIVES TUBES (ANY AMOUNT OF GAS) CONSTITUTE A POSITIVE PRESUMPTIVE TEST

TOTAL INCUBATION TIME FOR LACTOSE OR LTB IS 48 HRS. ± 3 HRS

INCUBATE BGLB TUBES FOR 48 HRS. ± 3 HRS. AT 35 DEG. C± 0.5 DEG. C.

INCUBATE EMB OR ENDO AGAR PLATES FOR 24 HRS. ± 2 HRS. AT 35 DEG. C± 0.5 DEG. C.

Part 3
LABORATORY METHODS FOR FECAL STREPTOCOCCUS
(Day-By-Day Procedures)

I GENERAL INFORMATION

A The same sampling and holding procedures apply as for the coliform test.

B The number of fecal streptococci in water generally is lower than the number of coliform bacteria. It is good practice in multiple dilution tube tests to start the sample planting series with one sample increment larger than for the coliform test. For example: If a sample planting series of 1.0, 0.1, 0.01, and 0.001 ml is planned for the coliform test, it is suggested that a series of 10, 1.0, 0.1, and 0.01 ml be planted for the fecal streptococcus test.

C Equipment required for the test is the same as required for the Standard Methods Presumptive and Confirmed Tests, except for the differences in culture media.

II STANDARD METHODS (Tentative) PROCEDURES

A First-Day Operations

1 Prepare the sample data sheet and labeled tubes of azide dextrose broth in the same manner as for the Presumptive Test. NOTE: If 10-ml samples are included in the series, be sure to use a special concentration (ordinarily double-strength) of azide dextrose broth for these sample portions.

2 Shake the sample vigorously, approximately 25 times, in an up-and-down motion.

3 Measure the predetermined sample volumes into the labeled tubes of azide dextrose broth, using the sample measurement and delivery techniques used for the Presumptive Test.

4 Shake the rack of tubes of inoculated culture media, to insure good mixing of sample with medium.

5 Place the rack of inoculated tubes in the incubator at 35° ± 0.5°C for 24 ± 2 hours.

B 24-hour Operations

1 Remove the rack of tubes from the incubator. Read and record the results from each tube. Growth is a positive test with this test. Evidence of growth consists either of turbidity of the medium, a "button" of sediment at the bottom of the culture tube, or both.

2 Label a tube of ethyl violet azide broth to correspond with each positive culture of azide dextrose broth. It may be permissible to use the same confirmatory transfer option as described for the coliform Confirmed Test, in this outline.

3 Shake the rack of cultures gently, to resuspend cells which have settled out to the bottom of the culture tubes.

4 Transfer three loopfuls or use a wood applicator to transfer culture from each growth-positive tube of azide dextrose broth to the correspondingly labeled tube of ethyl violet azide broth.

5 As transfers are made, place the newly inoculated tubes of ethyl violet azide broth in a separate rack while returning the AD tubes to their former positions in the rack.

6 Return the rack, all azide dextrose broth tubes and newly-inoculated tubes of ethyl violet azide broth, to the incubator. Incubate 24 ± 2 hours at 35° ± 0.5°C.

C 48-hour Operations

1 Remove the rack of tubes from the incubator. Read and report results. Growth, either in azide dextrose broth or in ethyl violet azide broth, is a positive test. Be sure to report the results of the azide dextrose broth medium under the "48" column for that medium and the results of the ethyl violet azide broth cultures under the "24" column for that medium.

2 Any 48-hour growth-positive cultures of azide dextrose broth are to be transferred (as before) to ethyl violet azide broth. Discard all 48-hour growth-negative tubes of azide dextrose broth and all 24-hour growth-positive tubes of ethyl violet azide broth.

3 Re-incubate the 24-hour growth-negative Ethyl Violet azide tubes after again re-inoculating with their respective positive Azide Dextrose tubes and the newly-inoculated tubes of ethyl violet azide broth 24 ± 2 hours at 35° ± 0.5°C.

D 72-hour Operations

1 Read and report growth results of all tubes of ethyl violet azide broth.

2 Discard all growth-positive cultures and all 48-hour growth-negative cultures.

3 Reincubate any 24-hour growth-negative cultures in ethyl violet azide broth after reinoculating with their respective positive azide dextrose tubes for an additional 24 ± 2 hours at 35° ± 0.5°C.

E 96-hour Operations

1 Read and report growth results of any remaining tubes of ethyl violet azide broth.

2 Codify results and determine fecal streptococci per 100 ml.

REFERENCES

1 Standard Methods for the Examination of Water and Wastewater (14th Ed). Prepared and published jointly by American Public Health Association, American Water Works Association, and Water Pollution Control Federation. 1975.

2 Geldreich, E.E., Clark, H.F., Kabler, P.W., Huff, C.B. and Bordner, R.H. The Coliform Group. II. Reactions in EC Medium at 45°C. Appl. Microbiol. 8:347-348. 1958.

3 Geldreich, E.E., Bordner, R.H., Huff, C.B., Clark, H.F. and Kabler, P.W. Type Distribution of Coliform Bacteria in the Feces of Warm-Blooded Animals. J. Water Pollution Control Federation. 34:295-301. 1962.

This outline was prepared by H. L. Jeter, Chief, Program Support Training Branch, USEPA, Cincinnati, Ohio 45268

Descriptors: Coliforms, Fecal Coliforms Fecal Streptococci, Indicator Bacteria, Laboratory Equipment, Laboratory Tests, Microbiology, Most Probable Number, MPN, Sewage Bacteria, Water Analysis

MEDIA AND SOLUTIONS FOR MULTIPLE DILUTION TUBE METHODS

I INTRODUCTION

A This chapter is intended to present detailed information on preparation and management of media and solutions needed with the tests and observations described elsewhere in this course manual.

B The preparation and management of supplies of culture media and solutions is one of the most critical aspects of a bacteriological water quality testing program.

1 In the same manner that the chemist relies on correctly prepared and standardized reagents for his analytical work, the bacteriologist must depend on satisfactory culture media for the type of analysis with which he is concerned.

2 In many laboratories preparation of media is entrusted to subprofessional personnel. Most such personnel, properly trained and guided, are able to perform the required tasks efficiently and reliably.

3 The professional supervisor should maintain close attention to all details, however, to guard against gradual introduction of bad habits in preparing and preserving media and other liquid supplies.

II GENERAL INFORMATION

A Use of Commercially Available Dehydrated Media

1 The preparation of all media described in this chapter is given in terms of the individual components, and preparation of the finished medium. This is done, even through commercially available dehydrated media are widely used, to acquaint the worker with the composition of the media and to indicate the required specifications of each medium.

2 The use of commercially available dehydrated media, requiring only careful weighing and dissolving of the powder in the proper quantity and quality of distilled water, is strongly recommended. Such media are much more likely to have uniformity at an acceptably high level of quality than are media compounded in the laboratory from the individual constituents.

3 It is recommended that the worker, when using commercially prepared dehydrated media, keep a careful record of the lot numbers of media being used. With first use of each new lot number of a given medium, it is suggested that the medium be checked for stability, pH after sterilization, and to see that performance is satisfactory. While rare, an occasional lot of medium will have some unforeseen fault which reduces or destroys its effectiveness. Maintenance of lot number records on medium gives opportunity for communication with the manufacturer to determine whether similar problems are being encountered in other laboratories.

B Quality of General Materials

1 Distilled water

Distilled water, or demineralized water, is required. It must be free from

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NOTE: Mention of commercial products and manufacturers is for illustration and does not imply endorsement by the Environmental Protection Agency.

dissolved metals or chlorine. Freedom from bactericidal constituents or growth promoting substances should be demonstrated through laboratory tests. A procedure for this test is described elsewhere in this course manual.

2 Beef extract

Any brand of beef extract is acceptable, provided that it is known to give results acceptable to the user. Meat infusion is not acceptable.

3 Peptones

Peptones are sold under a wide variety of trade names. Any peptone shown satisfactory by comparative tests with an acceptable peptone, may be accepted.

4 Sugars

All sugars must be chemically pure, and suitable for bacteriological media.

5 Agar

Any form of bacteriologic grade of agar can be used.

6 General chemicals must be reagent grade or ACS if used in culture media. Chemicals used in the distilled water quality test must be of the highest purity available.

7 Dyes

All dyes used in culture media must be certified by the Biological Stain Commission; they will be so labeled on the container.

C Quality of Equipment and Supplies Used for Preparation of Media

1 Glassware

It is recommended that all glassware be of borosilicate glass. Such glass is not subject to release of soluble products into the culture medium, as with some of the so-called "soft glass."

2 Balance

A balance with sensitivity of ± 2 grams with a load of 150 grams is the minimum acceptable standard for weighing of culture media in dehydrated form.

3 pH meter

An electrometric meter is recommended. While a comparator block with pH indicator solutions is useful for such media as lauryl tryptose broth, it cannot be used satisfactorily with dye-containing media such as brilliant green lactose bile broth. Therefore it is suggested that all pH control work on bacteriological media be done with an electrometric type of pH meter. Accuracy of the meter should be established through calibration against a standard buffer.

4 Autoclave

The autoclave should be of sufficient size to permit loose packing of tubed media when normal load is being sterilized. This is to permit free access of steam to all surfaces.

Operation should be such that sterilizing temperature is reached in not more than 30 minutes.

A pressure gauge should be present. More important, the autoclave should be equipped with at least 1 thermometer, which should be located properly in the exhaust line.

Pressure regulation should permit operation up to and including 121°C. When media containing carbohydrates are present, sterilization should be continued 12 - 15 minutes; in media not containing carbohydrates, normal sterilization time should be a standard 15 minutes.

After sterilization, media should be removed from the autoclave as soon as possible. In no case should an autoclave simply be turned off after the usual exposure to steam under pressure, and allowed to stand until the following morning before removing media.

5 Utensils for mixing and preparing media

Borosilicate glass is suggested, but other materials, such as stainless steel, porcelain (unchipped) containers, or other containers free of soluble bactericidal or bacteriostatic materials, are acceptable. In any case, the containers must be thoroughly clean.

III CONCENTRATION OF MEDIA

A Basic formulas of all media described in Section IV are presented as single-strength media. Most media are used in the single-strength concentration.

B The concentration of primary inoculation media (media into which the measured portions of the original sample are delivered) requires special consideration.

1 When the amount of medium is 10 ml or greater, and the volume of sample or sample dilution is 1 ml or less, then single-strength medium is satisfactory.

2 When the sample volume introduced into the primary inoculation medium is greater than 1 ml, then it is necessary to compensate for the diluting effect of the sample on the culture medium. In such cases, it is necessary to increase the initial concentration of the medium so that after sample inoculation the concentration of nutrients in medium-plus-sample is equivalent to the concentration of nutrients in the single strength medium.

IV PREPARATION OF MEDIA AND SOLUTIONS

A Lauryl Tryptose Broth (Lauryl Sulfate Broth)

1 Use: Primary inoculation medium in Presumptive Test

2 Composition:

Tryptose (or Trypticase or equivalent)	20.0	g
Lactose	5.0	g
Dibasic Potassium Phosphate (K_2HPO_4)	2.75	g
Monobasic Potassium Phosphate (KH_2PO_4)	2.75	g
Sodium Chloride	5.0	g
Sodium Lauryl Sulfate	0.1	g
(Total Dry Constituents	35.60	g)
Distilled Water	1000	ml

Sterilization: 12 - 15 minutes at 121°C
Reaction after sterilization: pH 6.8 approximately)

3 Compensation for diluting effect of samples

No. ml medium in tube	Ml of sample or dilution	Nominal concentration before inoculation	No. grams dehydrated medium per liter
10	0.1 - 1.0	1x	35.6
10	10	2x	71.2
20	10	1.5x	53.4
35	100	4x	137.3

B Brilliant Green Lactose Bile Broth

1 Use: Confirmed Test

2 Composition

Peptone (Bacto or equivalent)	10.0	g
Lactose	10.0	g
Oxgall (dehydrated)	20.0	g
Brilliant Green	0.0133	g
(Total weight dry constituents	40.0133	g)

Distilled Water	1000	ml

Sterilization: 12 - 15 minutes at 121°C
Reaction after sterilization: pH 7.1 to 7.4

C Eosin Methylene Blue Agar (Levine's Modification)

1 Confirmed Test

Use: Isolation of coliform-like colonies as a preliminary to Completed Test procedures.

2 Composition

Peptone (Bacto or equivalent)	10	g
Lactose	10	g
Dipotassium Phosphate (K_2HPO_4)	2	g
Agar	20	g
Eosin Y	0.4	g
Methylene Blue	0.65	g
(Total weight dry constituents	43.05	g)
Distilled Water	1000	ml

Sterilization: 12 - 15 minutes at 121° C

3 Special suggestions on preparation:

a This medium can be prepared and dispensed into bottles or flasks in portions of 100 ml or 200 ml each. The sterile medium may be stored for extended periods in cool places out of the light.

b When ready for use of such medium, the medium should be melted by immersion of the bottle of prepared medium in a boiling water bath, after which it is dispensed into sterile Petri dishes in portions of approximately 15 ml. After cooling and solidifying in the Petri dish, the medium is ready for use. It should be used preferably on the day it is poured into Petri dishes, but can be stored for a day or two in the refrigerator.

c An alternate method of preparing this medium requires preparation the agar base medium which includes all the constituents of the medium except the dyes. When ready to use such a preparation, the agar base medium is melted in a water bath, and to each 100 ml of the melted agar base medium, 2 ml of 2% of aqueous solution of eosin Y and 1.3 ml of 0.5% methylene blue solution is delivered with a pipet. The medium is mixed thoroughly, poured into Petri dishes, and used as previously described.

D Agar Slants

1 Use: This medium is used in the Completed Test, to cultivate pure cultures of strains of bacteria being cultivated in preparation of a Gram-stained smear.

2 Composition: The medium is nutrient agar

Peptone	5.0	g
Beef extract	3.0	g
Agar	15.0	g
(Total weight dry constituents	23.0	g)
Distilled Water	1000	ml

Sterilization: 15 minutes at 121° C

Reaction after sterilization: pH 6.8 approximately

3 Special instructions: Dissolve the constituents, using heat as needed; dispense in amounts of approximately 8 ml per tube. Screw-capped tubes extend shelf life of the medium. After sterilization, remove the melted medium from the autoclave and place in a slanting position until the medium has become solidified. A routine procedure should be established so that a uniform volume of medium and a uniform surface of slanted medium be present in each tube. While this has no particular bearing on Standard Methods procedures, certain other laboratory procedures do require uniform exposed surface area of the slanted medium.

E Plate Count Agar

1 Use: This medium is used in the distilled water test. It is not used in other Standard Methods procedures described in this course manual.

2 Composition: (Tryptone Glucose Yeast Agar)

Peptone-tryptone (or equivalent)	5.0 g
Yeast extract	2.5 g
Glucose (dextrose)	1.0 g
Agar	15.0 g
(Total weight dry constituents	23.5 g

Distilled Water 1000 ml

Sterilization: 15 minutes at 121°C

Reaction after sterilization: pH 7.0 ± 0.1

3 Special instructions in preparation: Use heat as needed to dissolve and melt the constituents. Dispense the medium in flasks or bottles in portions of 100 or 200 ml each and sterilize. In this state it can be preserved for many months, provided that it is protected from evaporation of the water.

When ready to use, melt the medium by heating, and cool to 45°C. At this temperature the medium still should be melted, and will be satisfacotry for preparation of pour plates for plate counts.

F EC Broth

1 Use: Test for fecal coliform bacteria

2 Composition:

Tryptose (Bacto or equivalent)	20.0 g
Lactose	5.0 g
Bile Salts (Bacto #3 or equivalent)	1.5 g
Dipotassium phosphate (K_2HPO_4)	4.0 g
Monopotassium phosphate (KH_2PO_4)	1.5 g
Sodium chloride	5.0 g
(Total weight dry constituents	37.0 g)

Distilled Water 1000 ml

Sterilization: 12 - 15 minutes at 121°C

Reaction after sterilization: pH 6.9

3 This medium is dispensed into culture tubes with inverted fermentation vials and suitable caps.

G Azide Dextrose Broth

1 Use: Primary inoculation medium for fecal streptococcal presumptive test.

2 Composition:

Beef extract	4.5 g
Tryptone or Polypeptone	15. g
Glucose	7.5 g
Sodium chloride	7.5 g
Sodium azide	0.2 g
(Total dry constituents	34.7 g)

Distilled Water 1000 ml

Sterilization: 12 - 15 minutes at 121°C

Reaction after sterilization: about pH 7.2

3 Fermentation vials are not used with azide dextrose broth.

H Ethyl Violet Azide Broth

1 Use: Confirmed test for fecal streptococci

2 Composition:

Tryptone or Biosate	20 g
Glucose	5 g
Sodium chloride	5 g
Potassium phosphate, diabasic (K_2HPO_4)	2.7 g
Potassium phosphate, monobasic (KH_2PO_4)	2.7 g
Sodium azide	0.4 g
Ethyl violet (certified dye if available)	.00083 g
(Total dry constituents	35.8 g)

Distilled Water 1000 ml

Sterilization: 12 - 15 minutes at 121°C

Reaction after sterilization: about pH 7

3 Fermentation vials are not used with ethyl violet azide broth.

I Buffered Dilution Water

1 Use: Preparation of sample dilutions preliminary to primary inoculation, in membrane filter work, and in plate counts.

2 Composition

a Stock phosphate buffer solution

Monobasic Potassium Phosphate (KH_2PO_4)	34.0 g
Distilled Water	500 ml

1N NaOH solution (about 175 ml) to give pH 7.2

Distilled water sufficient to bring final volume to 1000 ml

b Working solution of phosphate buffered distilled water

Stock phosphate buffer solution	1.25 ml
Distilled water	1000 ml

3 Preparation and handling:

a Stock solution: After preparation the stock solution should be stored in the refrigerator until use. If at any time evidence of mold or other contamination appears, the stock solution should be discarded and a fresh solution prepared.

b Working solution: Dispense the required amount into distilled water, and deliver into screw-capped bottles for dilution water. The amount added should be such that, after sterilization, the bottles will contain 99 ± 2 ml of the dilution water. Ordinarily this requires initial addition of approximately 102 ml of the solution prior to sterilization.

c Sterilization is 20 minutes at 121°C.

d Tightly stoppered bottles of the dilution water, protected against evaporation, in suitable containers, appear to last indefinitely.

J Solutions for Gram Stain

1 Ammonium oxalate crystal violet solution:

a Dissolve 2 g crystal violet (approximately 85% dye content) in 20 ml of 95% ethyl alcohol.

b Dissolve 0.8 grams ammonium oxalate in 80 ml distilled water.

c Mix solutions a and b.

d Filter through cheesecloth or coarse filter paper.

e Problems with the gram stain technique frequently are traceable to the ammonium oxalate crystal violet solution. In the event that decolorization does not seem satisfactory, the amount of crystal violet in the solution can be reduced to as little as 10% of the recommended amount.

2 Lugol's iodine: Dissolve 1 g iodine crystals and 2 g potassium iodide in the least amount (usually about 5 ml) of distilled water in which they are soluble. After all crystals are in solution, add sufficient distilled water to bring the final solution to a volume of 300 ml.

3 Counterstain: Dissolve 2.5 grams of safranin in 100 ml of 95% ethyl alcohol. For the working solution of counterstain, add 10 ml of this solution of safranin to 100 ml of distilled water.

REFERENCES

1 Standard Methods for the Examination of Water and Wastewater. 14th ed. 1975.

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Descriptors: Cultures, Bacteria, Enteric Bacteria, MPN, Multiple Dilution Method, Most Probable Number, Coliform, Fecal Coliform

USE OF TABLES OF MOST PROBABLE NUMBERS

Part 1

I INTRODUCTION

A Using probability mathematics, it is possible to estimate the number of bacteria producing the observed result for any combination of positive and negative results in dilution tube tests. Because the computations are so repetitious and time-consuming, it is common laboratory practice to use Tables of Most Probable Numbers. These tables are orderly arrangements of the possible cultural results obtainable from inoculating various sample increments in differential culture media. Each possible combination of positive and negative tube results is accompanied by the result (MPN) of the calculated estimate and the 95% confidence limits of the MPN.

B The Tables of Most Probable Numbers used in the current (14) edition of Standard Methods for the examination of Water and Wastewater were developed by Swaroop.(1) Previous editions of Standard Methods have used the tables prepared by Hoskins.(2)

1 Most of the tables are based on using 3 sample volumes in decreasing decimal increments. Thus, the systems are based on using volumes of 10 ml, 1.0 ml, and 0.1 ml, etc. Other quantity relationships can be used, such as 50 ml, 10 ml, and 1.0 ml in a table. Tables of Most Probable Numbers can be prepared for any desired series of sample increments.

2 In addition, tables can be devised for different numbers of replicate inoculations of individual sample volumes. For example, the MPN Table most commonly used in the laboratories of this agency is based on five replicate 10 ml portions, five 1.0 ml portions, and five 0.1 ml portions. A separate table is required for another combination of sample volumes, consisting of five replicate 10 ml portions, one 1.0 ml portion, and one 0.1 ml portion. This is popular in bacteriological potability tests on water. MPN Tables can be prepared for any desired combinations of replicates of the sample increments used in a dilution tube series.

3 An approximation of the MPN values shown in the Tables can be obtained by a simple calculation, developed by Thomas.(3) The formula and application of this calculation is shown on a later page of this chapter.

C The method of using a Table of Most Probable Numbers is described here, based on the table for five 10 ml portions, five 1.0 portions, and five 0.1 portions. The principles apply equally to the other tables presented in the current edition of Standard Methods for the Examination of Water and Wastewater.

II DETERMINING THE MOST PROBABLE NUMBER

A Codifying Results of the Dilution Tube Series

If five 10 ml portions, five 1.0 ml portions, and five 0.1 ml portions are inoculated initially, and positive results are secured from five of the 10 ml portions, three of the 1.0 ml portions and none of the 0.1 ml portions, then the coded result of the test is 5-3-0. The code can be looked up in the MPN Table, and the MPN per 100 ml is recorded directly. If more than the above three sample volumes are to be considered, then the determination of the coded result may be more complex. The examples described in Table 1 are useful guides for selection of the significant series of three sample volumes.

Table 1. EXAMPLES OF CODED RESULTS

No. ml sample per tube →	100	10	1.0	0.1	0.01	0.001	Code	See Below
No. tubes per sample vol. →	5	5	5	5	5	5		
No. tubes in sample giving positive results in test		5	4	1			5-4-1	
	5	5	4	0	0	0	5-4-0	(1)
		4	1	0	0	0	4-1-0	(2)
	5	5	4	1	1	0	5-4-2	(3)
		5	5	5	4		5-5-4	(4)
		5	5	5	5		5-5-5	(5)
		0	0	0	0		0-0-0	(6)
		0	1	0	0		0-1-0	(7)
		1	0	0	0		1-0-0	(8)

Discussion of examples:

1 When all the inoculated tubes of more than one of the decimal series give positive results, then it is customary to select the smallest sample volume (here, 10 ml) in which all tubes gave positive results. The results of this volume and the next lesser volumes are used to determine the coded result.

2 When none of the sample volumes give positive results in all increments of the series, then the results obtained are used to designate the code. Note that it is not permissible to assume that if the next larger increment had been inoculated, all tubes probably would have given positive results and therefore assign a 5-4-1 code to the results.

3 Here the results are spread through four of the sample volumes. In such cases, the number of positive tubes in the smallest sample volume is added to the number of tubes in the third sample volume (counting down from the smallest sample volume in which all tubes gave positive results).

4 Here it is necessary to use the 5-5-4 code, because inoculations were not made of 0.001 ml sample volumes; and it is not permissible to assume that if such sample volumes had been inoculated, they would have given negative results, or any other arbitrarily-designated result.

5 This is an indeterminate result. Many MPN tables do not give a value for such a result. If the table used does not have the code, then look up the result for code 5-5-4, and report the result "greater than" the value shown for the 5-5-4 code. The first number of the 5-5-4 code is based on the 1.0 ml sample volume.

6 Like (5), this is an indeterminate result. If the code does not appear in the table being used, then look up the result for code 1-0-0, and report the MPN as "less than" the value shown for the 1-0-0 code.

7 The current edition of Standard Methods stipulates this type of code designation when unusual results such as this occur.

8 Note the difference from (7) above. Inoculations of 100 ml portions were not made, and it cannot be assumed that the result would have called for code 0-1-0.

B Computing and Recording the MPN

When the dilution tube results have been codified, they are read and recorded from the appropriate MPN Table.

1 If, as in the first four of the examples shown under (A) the first number in the coded result represents a 10 ml sample volume, then the MPN per 100 m is read and recorded directly from the appropriate column in the table.

2 On the other hand, if the first number in the coded result represents a sample volume other than 10 ml, then a calculation is required to give the corrected MPN. For example (4) under (A) above, the first "5" of the 5-5-4 code represents a sample volume of 1.0 ml. Look up the 5-5-4 code as if the 1.0 ml volume actually were 10 ml, as if the 0.1 ml volume actually were 1.0 ml and as if the 0.01 ml volume actually were 0.1 ml. The MPN obtained (1600) then is multiplied by a factor of 10 to give the corrected value. A simple formula for this type of correction is shown on a later page of this chapter.

III PRECISION OF THE MPN VALUE

A The current edition of Standard Methods shows for each MPN value, the 95% confidence limits for that value. This draws attention to the fact that a given MPN value is not a precise measurement, but an estimate. The 95% confidence limits means that the observer will be correct 95% of the time when he considers that the actual number of cells producing the observed combination of positive and negative tubes was somewhere between the stated upper limit and the stated lower limit.

B The greater the number of replicates of each sample volume in a dilution series, the greater the precision (in other words, the narrower the limits of the 95% confidence range) of the test. The precision of results, based on numbers of tubes inoculated per sample volume, is shown in Table 2.

C Woodward (4) and other workers have studied the precision of the MPN in detail. Such reports should be studied by those desiring further information regarding the precision of the MPN test.

Table 2. Approximate Confidence Limits for Bacterial Densities as Per Cent of MPN as Determined from Various Numbers of Tubes in Three Decimal Dilutions*

Number of tubes	50%		75%		80%		90%		95%	
in each dilution	Lower	Upper	Lower	Upper	Lower	Upper	Lower	Upper	Lower	Upper
1	33	186	18	340	15	402	10	637	6.5	955
2	47	160	31	246	27	276	20	383	15	511
3	53	150	38	215	34	237	26	311	21	395
5	64	139	49	182	46	196	37	241	31	289
10	76	127	63	152	60	160	52	184	46	208

*The interpretation of these figures is as follows: When MPN estimates are made on the basis of dilution tests using one tube in each of three decimal dilutions, you will be right 50% of the time if you say that the true bacterial density is between 33% and 186% of the MPN. If you had used 5 tubes in each dilution you could reduce this interval to from 64% to 139% of the MPN and still be right 50% of the time. If a greater certainty were desired, say 95%, you would have to widen this interval to from 31% to 289%.

IV OCCURRENCE OF IMPROBABLE TUBE RESULTS

A Many of the theoretically possible tube results are omitted from the MPN Table. For example, codes 0-0-3, 0-0-4, and 0-0-5 are not included as well as many others. These are omitted, because, in the opinion of the authors of the tables, the probability of occurrence of such results is so low as to exclude them from practical consideration.

B The frequency of occurrence of various code results is shown in the Table 2 both on a theoretical basis and on the basis of actual laboratory experience.

C From the MPN tables, it can be inferred that the codes omitted from the MPN Table can be expected to occur up to 1% of the time. If, in reviewing laboratory data, the theoretically unlikely codes occur appreciably more than 1% of the time, there is an indication for inquiry into the causes. Such results can occur (1) as a consequence of faulty laboratory procedures, or (2) as a result of extraneous influences in the samples.

D The current edition of Standard Methods does not include MPN values for many rare combinations listed in previous editions. By pruning out those codes listed as Group IV in Table 3, the table has been considerably condensed. Table 4 suggests maximum permissible numbers of samples for various numbers of samples tested.

Table 3

FIVE-TUBE AND THREE-TUBE CODES THAT INCLUDE 99 PER CENT OF ALL RESULTS

Group	Theoretically Expected Percentage of Results	Theoretically Expected Cumulative Percentage	Observed Percentage of 360 Samples
		Five-Tube-Test	
Class 1 codes 550, 551, 552, 553, 554, 500, 510, 520, 530, 540, 100, 200, 300, 400.	67.5	67.5	68.0
Class 2 codes 511, 521, 531, 541, 542, 110, 210, 310, 410, 420.	23.6	91.1	23.1
Class 3 codes 501, 010, 532, 320, 522, 220, 543, 430, 120, 533, 330, 502, 020, 544, 440, 301, 401, 431, 201, 411, 101, 311, 421, 211, 001.	7.9	99.0	7.5
Improbable codes	1.0	100.0	1.4
		Three-Tube Test	
Class 1 codes 330, 331, 332, 300, 310, 320, 100, 200.	81.5	81.5	81.7
Class 2 codes 321, 311, 301, 210, 110, 010.	14.9	96.4	14.1
Class 3 codes 322, 220, 201, 101 312, 120.	2.7	99.1	3.7
Improbable codes	0.9	100.0	0.6

Table 4

MAXIMUM PERMISSIBLE NUMBERS OF IMPROBABLE CODES FOR VARIOUS NUMBERS OF SAMPLES TESTED

Number of Samples	Maximum Number of Improbable Codes
1 - 15	1
16 - 45	2
46 - 83	3
84 - 130	4
131 - 180	5
181 - 233	6
234 - 290	7
291 - 350	8
351 - 413	9
414 - 477	10
478 - 543	11

F Table 5 is from International Standards for Drinking-Water, published by the World Health Organization, Geneva (1958). The last three values, not shown in the WHO publication, are from Woodward, "How Probable is the Most Probable Number."(4)

F Several theoretically possible combinations of positive tube results are omitted in Table 5. These combinations are omitted because the statistical probability of occurrence of any of the missing results is less than 1%. If such theoretically unlikely tube combinations occur in more than 1% of samples, there is need for review of the laboratory procedures and of the nature of the samples being tested.

When the series of decimal dilutions is other than 10, 1.0 and 0.1 ml, use the MPN in Table 5, according to the following formula:

$$\underset{\text{(from table)}}{\text{MPN}} \times \frac{10}{\text{Largest quantity tested}}$$

$$= \text{MPN/100 ml}$$

Example: From a sample of water, 5 out of five 0.01 - ml portions, 2 out of five 0.001 - ml portions, and 0 out of five 0.0001 - ml portions, gave positive reactions.

From the code 5-2-0 in the MPN table, the MPN index is 49

$$\underset{\text{(from table)}}{49} \times \frac{10}{0.01} = 49,000$$

MPN Index = 49,000

A simple approximation of the most probable number may be obtained from the following formula (after Thomas):

MPN/100 ml =

$$\frac{\text{No. of Positive Tubes} \times 100}{\sqrt{\text{No. of ml in negative tubes)} \times \text{(No. of ml in all tubes)}}}$$

Example: From a sample of water, 5 out of five 10 - ml portions, 2 out of five 1.0 ml portions, and 0 out of five 0.1 ml portions gave positive results.

$$\text{MPN/100 ml} = \frac{7 \times 100}{\sqrt{(3.5) \times (55.5)}} = 50.22$$

MPN/100 ml = 50

Note that the MPN obtained from the table on the preceding pages with these tube results is 49. "Most probable numbers computed by the above formula deviate from values given by the usual methods by amounts which ordinarily are insignificant. The formula is not restricted as to the number of tubes and dilutions used ---" (Thomas)

Table 5. MPN INDEX AND 95% CONFIDENCE LIMITS FOR VARIOUS COMBINATIONS OF POSITIVE AND NEGATIVE RESULTS WHEN FIVE 10-ML PORTIONS, FIVE 1-ML PORTIONS AND FIVE 0.1 ML PORTIONS ARE USED

No. of Tubes Giving Positive Reaction out of			MPN Index per 100 ml	95% Confidence Limits		No. of Tubes Giving Positive Reaction out of			MPN Index per 100 ml	95% Confidence Limits	
5 of 10 ml Each	5 of 1 ml Each	5 of 0.1 ml Each		Lower	Upper	5 of 10 ml Each	5 of 1 ml Each	5 of 0.1 ml Each		Lower	Upper
0	0	0	<2								
0	0	1	2	<0.5	7	4	2	1	26	9	78
0	1	0	2	<0.5	7	4	3	0	27	9	80
0	2	0	4	<0.5	11	4	3	1	33	11	93
						4	4	0	34	12	93
1	0	0	2	<0.5	7						
1	0	1	4	<0.5	11	5	0	0	23	7	70
1	1	0	4	<0.5	11	5	0	1	31	11	89
1	1	1	6	<0.5	15	5	0	2	43	15	110
1	2	0	6	<0.5	15	5	1	0	33	11	93
						5	1	1	46	16	120
2	0	0	5	<0.5	13	5	1	2	63	21	150
2	0	1	7	1	17						
2	1	0	7	1	17	5	2	0	49	17	130
2	1	1	9	2	21	5	2	1	70	23	170
2	2	0	9	2	21	5	2	2	94	28	220
2	3	0	12	3	28	5	3	0	79	25	190
						5	3	1	110	31	250
3	0	0	8	1	19	5	3	2	140	37	340
3	0	1	11	2	25	5	3	3	180	44	500
3	1	0	11	2	25	5	4	0	130	35	300
3	1	1	14	4	34	5	4	1	170	43	490
3	2	0	14	4	34	5	4	2	220	57	700
3	2	1	17	5	46	5	4	3	280	90	850
3	3	0	17	5	46	5	4	4	350	120	1,000
4	0	0	13	3	31	5	5	0	240	68	750
4	0	1	17	5	46	5	5	1	350	120	1,000
4	1	0	17	5	46	5	5	2	540	180	1,400
4	1	1	21	7	63	5	5	3	920	300	3,200
4	1	2	26	9	78	5	5	4	1600	640	5,800
4	2	0	22	7	67	5	5	5	>2400		

Table 6. MPN AND 95% CONFIDENCE LIMITS FOR VARIOUS COMBINATIONS OF POSITIVE RESULTS IN A PLANTING SERIES OF FIVE 10-ml PORTIONS OF SAMPLE

No. of Positive Tubes Out of:	MPN per	Limits of MPN	
Five 10-ml Tubes	100 ml	Lower	Upper
0	2.2	0	6.0
1	2.2	0.1	12.6
2	5.1	0.5	19.2
3	9.2	1.6	29.4
4	16.0	3.3	52.9
5	> 16	8.0	

Table 7. MPN AND 95% CONFIDENCE LIMITS FOR VARIOUS COMBINATIONS OF POSITIVE RESULTS IN A PLANTING SERIES OF FIVE 10-ml, ONE 1-ml, AND ONE 0.1-ml PORTIONS OF SAMPLE

No. of Positive Tubes Out of:			MPN per 100 ml	Limits of MPN	
Five 10-ml Tubes	One 1-ml Tube	One 0.1-ml Tube		Lower	Upper
0	0	0	<2		5.9
0	1	0	2	0.050	13
1	0	0	2.2	0.050	13
1	1	0	4.4	0.52	14
2	0	0	5	0.54	19
2	1	0	7.6	1.5	19
3	0	0	8.8	1.6	29
3	1	0	12	3.1	30
4	0	0	15	3.3	46
4	0	1	20	5.9	48
4	1	0	21	6.0	53
5	0	0	38	6.4	330
5	0	1	96	12	370
5	1	0	240	12	3700
5	1	1	>240	88	

IV TABLES OF MOST PROBABLE NUMBERS

These tables consist of the MPN indices and 95% confidence limits, within which the actual number of organisms can lie, for various combinations of positive and negative tubes. Three MPN tables are presented. Table 5 is based on five 10 ml five 1.0 ml and five 0.1 ml sample portions. Table 6 is based on five 10 ml sample portions; and Table 7 is based on five 10 ml, one 1 ml and one 0.1 ml sample portion.

REFERENCES

1 Swaroop, S. Numerical Estimation of *B. coli* by Dilution Method. *Indian J. Med. Research.* 26:353. 1938.

2 Hoskins, J.K. Most Probable Numbers for Evaluation of Coli-Aerogenes Tests by Fermentation Tube Method. Public Health Reports. 49:393. 1934.

3 Thomas, H. Al, Jr. Bacterial Densities from Fermentation Tubes. J.A.W.W.A. 34:572. 1942.

4 Woodward, R.L. How Probable is the Most Probable Number? J.A.W.W.A. 49:1060. 1958.

5 *Standard Methods for the Examination of Water and Wastewater.* 14th Edition. Prepared and Published Jointly by American Public Association. American Water Works Association, and Water Pollution Control Federation.

This outline was prepared by H. L. Jeter, [illegible]tor, National Training Center, Water Programs Operations, Environmental Protection Agency, Cincinnati, OH 45268.

MPN THEORY

Part 2

I DERIVATION OF THE MPN

A Assumptions

The validity of the MPN procedure is based upon two principal assumptions.

1 In statistical language, the first is that the organisms are distributed randomly throughout the liquid. This means that an organism is equally likely to be found in any part of the liquid, and that there is no tendency for pairs or groups of organisms either to cluster together or to repel one another.

2 The second assumption is that each sample from the liquid, when incubated in the culture medium, is certain to exhibit growth whenever the sample contains one or more organisms.

B The Probability Equation

Based upon these assumptions, an equation for the probability of the observed combination of positive and negative tubes can be derived as a function of the true density δ. By solving this equation for different values of δ a curve can be plotted as shown in Figure 1.

Curves of this type always have a single maximum or peak. The value of δ, say d, which corresponds to the peak of the curve is called the most probable number, commonly designated as MPN.

The MPN is "most probable" in the sense that it is the number which maximizes the probability of the observed results. It is interesting to note that although the original derivation of the MPN predates modern statistical estimation, the MPN procedure corresponds to the currently accepted estimation procedure known as the "method of maximum likelihood."

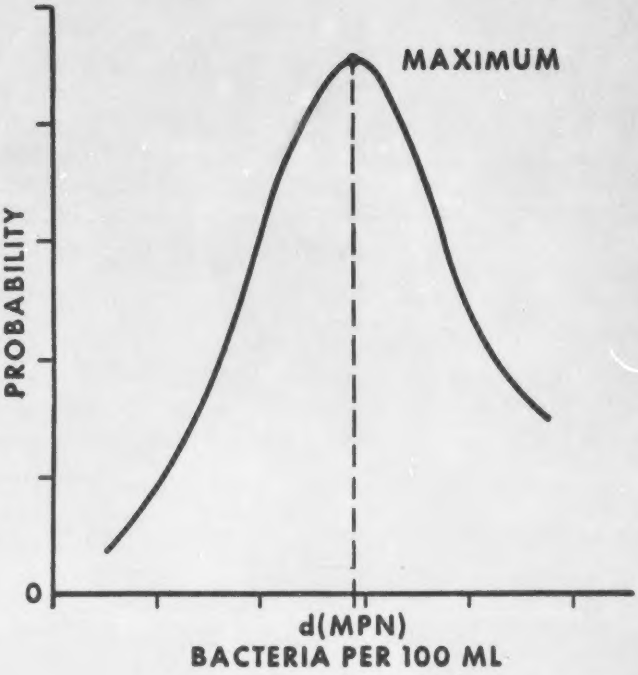


FIGURE 1

C Indeterminant Solutions

The MPN provides a meaningful estimate of δ only if there are both positive and negative tubes in at least one dilution. If all tubes are negative, the maximum of the probability curve occurs when δ is set equal to zero (see Figure 2) and thus the MPN is zero. If all tubes are positive, the maximum of the probability curve occurs when δ is set equal to infinity (see Figure 3) and thus the MPN is infinity.

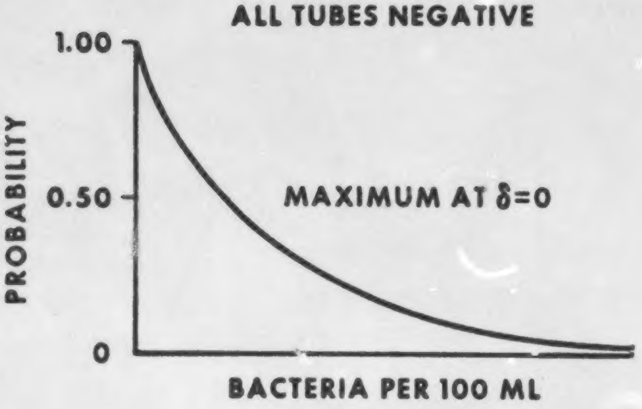


FIGURE 2

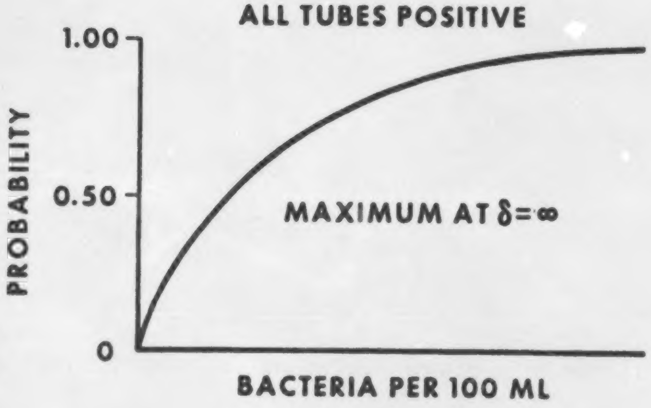


FIGURE 3

II DISTRIBUTION OF MPN VALUES

A Skewed Distribution

If a very large number of independent MPN determinations were made on the same water sample, the distribution of the MPN values would be such that very high values relative to the median value would occur more frequently than very low values. Thus the distribution of MPN values is skewed to the right as shown in Figure 4.

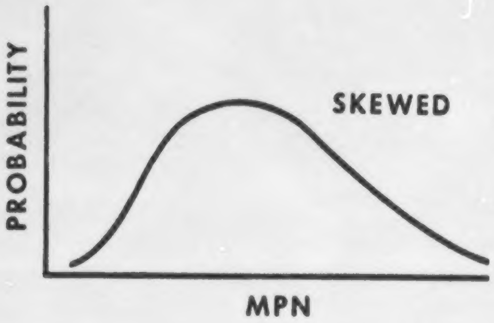


FIGURE 4

B Logarithmically Normal Distribution

Since it is mathematically inconvenient to work with data distributed asymmetrically, it is desirable to transform the skewed data in such a way that the transformed values have a symmetric distribution resembling the normal. In the case of MPN values the logarithms of the MPN's are approximately normally distributed as shown in Figure 5.

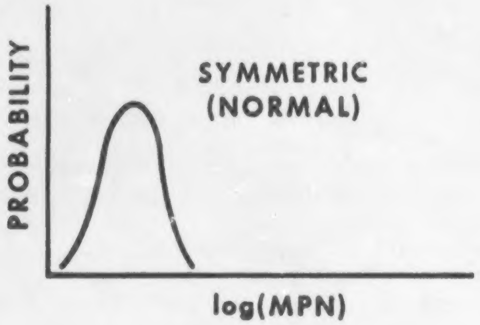


FIGURE 5

C Precision of MPN Estimates

The lack of precision of MPN estimates of bacterial densities is generally recognized. A measure of the precision is given by the confidence limits on the estimate which can be computed on the basis of the normal distribution of the logarithms of the MPN values. It has been verified that three-tube and five-tube MPN estimates are approximately logarithmically normal and the standard deviation of the logarithms of the MPN's is given by the formula:

$$\sigma_{\log} = \frac{0.58}{\sqrt{n}}$$

where $\sigma_{\log}$ is the standard deviation of the logarithms of the MPN estimates and n is the number of tubes in each dilution.

The upper and lower 95% confidence limits of an MPN estimate are given by the formulas:

$$\begin{aligned} UCL &= \text{antilog}\,(\log MPN + 1.96\sigma_{\log}) \\ &= MPN \cdot k, \\ LCL &= \text{antilog}\,(\log MPN - 1.96\sigma_{\log}) \\ &= MPN \div k, \end{aligned}$$

where $k = \text{antilog}\ (1.96\sigma_{\log})$.

Notice that the confidence limits are not symmetric about the MPN estimate.

The precision of the MPN estimate can be increased by increasing the number of tubes per dilution. Figure 6 shows the width of the 95% confidence interval expressed as a percentage of the MPN estimate for various values of n. Notice that the width of the confidence interval decreases as n increases.

III PLANNING A DILUTION SERIES

A The Rationale

It was mentioned that the MPN procedure provides a reasonable estimate of the true density only if there are both positive and negative tubes in at least one dilution. It follows that in a series of dilutions the expected number of organisms in the highest sample volume (lowest dilution) ν_H should be at least one, otherwise all tubes may be negative and the result will be an indeterminant value.

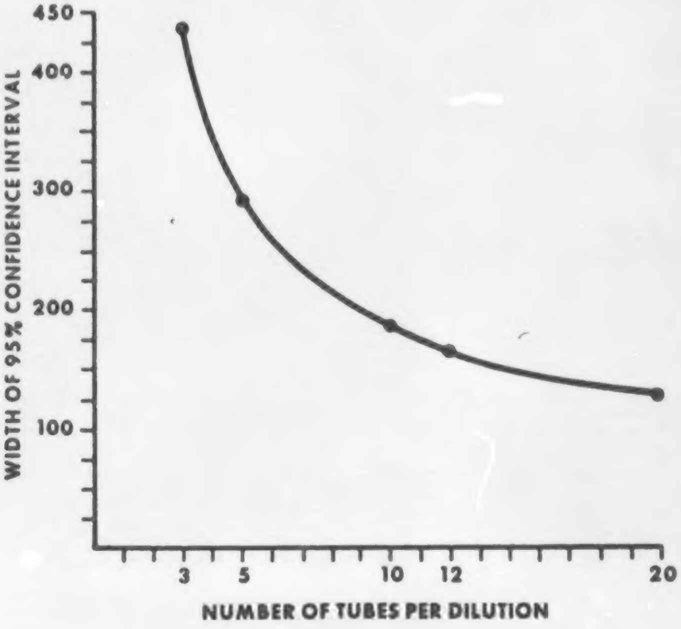


FIGURE 6

Similarly, the expected number or organisms in the lowest sample volume (highest dilution) ν_L should not exceed one, to avoid the risk that all tubes will be positive.

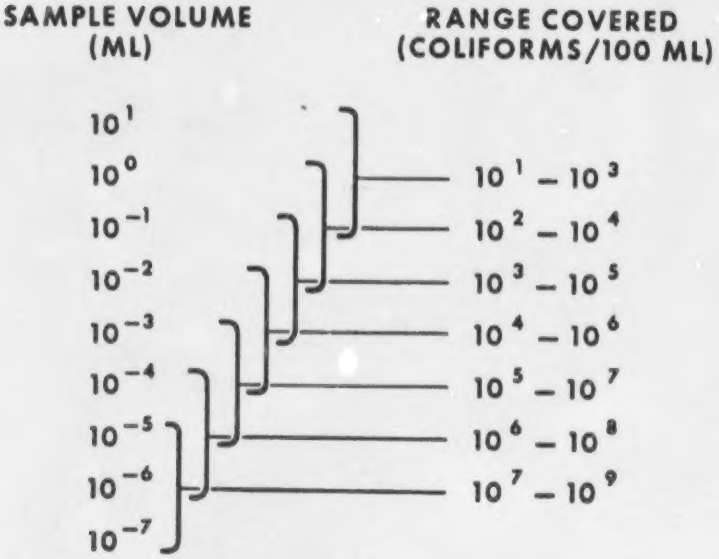
B The Rule

The above line of reasoning leads to the rule that a dilution series is capable of estimating any density between $1/\nu_H$ and $1/\nu_L$. In practice, we use the rule by first guessing two limits δ_H and δ_L between which we are fairly certain that the actual density lies. The sample volumes are then chosen to satisfy the rules

$$\nu_H \geq \frac{1}{\delta_L} \; ; \; \nu_L \leq \frac{1}{\delta_H} .$$

Table 1 displays the range of densities covered by various decimal dilution series.

TABLE 1



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Descriptors: Bacteria, Coliforms, MPN, Most Probable Number, Measurement, Microbiology, Laboratory Tests

THE MEMBRANE FILTER IN WATER BACTERIOLOGY

I HISTORICAL BACKGROUND

There is sometimes a tendency to look upon membrane filters and their bacteriological applications as new developments. Both the filters and many of their present bacteriological applications are derived from earlier work in Europe.

A Some European developments prior to 1947 are as follows:

1 Fick is credited with application of collodion membranes in biological investigations in 1855.

2 Sanarelli, in 1891, reported development of membrane filters impermeable to bacteria but permeable to their toxins.

3 Bechhold, in the early 1900's made a systematic study of the physicochemical properties of a number of varieties of these membranes. After 1911 numerous investigations were made in several countries with respect to the properties of collodion membranes.

4 Zsigmondy and Bachmann, 1916-1918, developed improved production methods which were applicable on a commercial scale. Membrane filters have been produced for many years at the Membranfiltergesellschaft, Sartorious Werke, in Goettingen, Germany. In 1919 Zsigmondy applied for a U.S. patent on his production methods; it was granted in 1922.

5 In the 1930's, W. J. Elford in England, and P. Graber in France, made new contributions in developing and teaching methods for making collodion membranes with controlled pore size.

6 Before World War II filtration procedures using the Zsigmondy membrane had been suggested for the determination of bacterial counts, coliform determinations, and isolation of pathogenic bacteria from water and other fluids. Most early interest in developing these techniques seems to have been in Germany and in Russia. During World War II Dr. G. Mueller applied membrane filter techniques to the bacteriological examination of water, following bomb destruction of many of the laboratories.

B Developments in the United States

1 In 1947, Dr. A. Goetz reported on a mission to Germany as a scientific consultant to the Technical Industrial Intelligence Branch, U. S. Department of Commerce. He obtained detailed information about the nature, method of preparation, and specific bacteriological applications of the Zsigmondy Membranfilter being manufactured by the Membranfiltergesellschaft in Goettingen.

2 After his return to this country, Dr. Goetz developed methods for preparing and improved type of membrane filter from domestic materials. On a small scale he manufactured filters under a government contract; afterward membrane filter manufacture was continued by a commercial organization.

3 In 1950, bacteriologists of the Public Health Service began intensive study of the applications of membrane filters in bacteriological examinations of water. Their first report was published in 1951, and was followed by numerous reports of other similar investigations. Such studies have been widely expanded, as indicated in references shown elsewhere in this manual.

NOTE: Mention of commercial products and manufacturers does not imply endorsement by the OWP, Environmental Protection Agency.

4 In 1955 the 10th Edition of "Standard Methods for the Examination of Water, Sewage, and Industrial Wastes" included a tentative method for coliforms by membrane filter method. In the 11th and 12th editions, the membrane filter method for coliforms has become official. In addition, methods for enterococcus (fecal streptococci) are included as tentative methods. The 13th edition had given the fecal streptococcus test a standard designation and the tentative method status reserved for the "pour plate" technique of quantitation.

5 The membrane filter is an official method for examination of potable waters in interstate commerce. The Public Health Service Drinking Water Standards (1962) state "Organisms of the coliform group... All the details of technique ...shall be in accordance with Standard Methods for Examination of Water and Wastewater, current edition..." Thus, acceptance by Standard Methods as official automatically validates a method for use with interstate waters.

II PROPERTIES OF MEMBRANE FILTERS

Membrane filters used in water bacteriology are flat, highly porous, flexible plastic discs about 0.15 millimeters in thickness and usually 47-50 millimeters in diameter.

A Principle of Manufacture

The procedures described below are from FIAT Report 1312. While the methods indicated by Goetz do not necessarily describe the current manufacturing processes, it is assumed that similar principles of manufacture still apply.

1 One or more cellulose esters, such as cellulose nitrate, is dissolved in a suitable solvent.

2 Water, or some other liquid insoluble in the cellulose solution, is added and mixed, to form an emulsion having great uniformity in size and distribution of droplets of the insoluble liquid.

3 The emulsion is cast on plates and dried in an environment rigidly controlled as to humidity and temperature. The droplets of insoluble fluid retain their size and identity in the dried film, eventually becoming the pores of the finished membrane.

4 The dried porous film is cut into filter discs of the desired size. Representative discs are subjected to control tests for accurate determination of the pore size obtained.

5 Particle retention by membrane filters is at or very near the filter surface, by a mechanical, sieve-like action. (This applies to hydrosols, not to aerosols.) Through manufacturing control it is possible to make membrane filters with controlled pore size, within narrow limits

B Some Important Characteristics of Membrane Filters

1 The membrane filters used in microbiology should be flat, circular, gridded, of uniform thickness and porosity, nontoxic to microorganisms, wettable, able to withstand commonly employed sterilizing conditions, and unaffected by the fluid to be filtered.

2 Without reference to specific manufacturers, some particulars of their products have included:

a ...Average pore diameter ranging from 5 millimicrons to 10 microns. Thicknesses ranging from 70 to 150 microns. Can be sterilized by autoclaving at 121°C for 10 minutes.

b ...mean flow pore size ranging from 7.5 millimicrons to 5 microns. The pore size used in water bacteriology having a standard diameter, has a water flow rate of 70cc/min/cm^2 and must pass 100 ml of particle-free water within 9 seconds.

c ... currently produced in more than twenty distinct pore sizes from 14 microns to 10 millimicrons in discs ranging from 13 mm to 293 mm in diameter. The total range of pore size distribution of the type used in water microbiology of 0.45 microns is plus or minus 0.02 micron.

d ... membranes are offered in graduated pore sizes ranging from 12 microns to 5 millimicrons. The types used in water bacteriology have a distilled water flow rate of 65 $ml/min/cm^2$ at 700 mm Hg differential pressure or an air flow rate of 0.4 $liters/min/cm^2$ at a differential pressure of 500mm water.

4 Membrane filters are wettable. Thus, after sample filtration, when a filter is placed on moist culture medium the medium diffuses through the pores and is available to organisms collected on the opposite surface.

5 Membrane filters are free of soluble chemical substances inhibitory to bacterial growth. Water soluble plasticizers are included in one commercially produced filter (glycerol, 2.5%). The cellulose esters themselves have some absorbing tendency illustrated by some dyes and heavy metals. Total ash is very low, less than 0.0001%.

6 Membrane filters have a uniform index of refraction. With membrane filters, this index is N_a: 1.5. When wetted with a liquid having refractive index within this range, the filters become transparent. This property permits direct microscopic examination of particulate matter collected on the filter surface.

7 Temperature resistance depends on plastics used in the filter. The nitrocellulose membrane filter is stable dry up to 125°C in air. Membranes of cellulose triacetate are advertised to withstand dry heat to 266°C. In general however, membranes in current use must be sterilized cautiously. Consult the laboratory equipment discussion for details. Overheating of all types interferes with filtration by blocking pores.

C Nomenclature

Membrane filters used in bacteriological tests on water are known under several names. Though the names are different, the filters are similar in form, properties, and method of use. Names commonly encountered are:

1 Membrane filter. This is the general name for filters made according to the general principles and having the properties discussed above. The term "membrane filter" is most used in technical reports on filters of this type.

2 Molecular filter. This name used by Goetz for the improved type of filter that he and his associates developed after study of the manufacturing methods at the Membranfiltergesellschaft in Goettingen, Germany.

3 Millipore filter is a trade name for membrane filters made by the Millipore Filter Corporation.

4 Bac-T-Flex filter is a trade name applied to certain membrane filters made by Carl Schleicher and Schuell Company.

5 Oxoid filter is a trade name applied to filters made by Oxo, Ltd., London, England.

6 Micropore, Polypore and Metricel have been trade names used by the Gelman Instrument Company.

III APPLICATIONS IN WATER BACTERIOLOGY

A The basic cultural procedures for bacteriological tests on membrane filters are:

1 A sample is filtered through a membrane filter.

2 The filter is placed in a culture container, on an agar medium or a paper pad impregnated with moist culture medium.

3 The inoculated filter is incubated under prescribed conditions of time, temperature, and humidity.

4 After incubation, the resulting culture is examined and necessary interpretations and/or additional tests are made.

B With variations in such factors as culture media, incubation time, and combinations with other cultural and biochemical tests, several different kinds of tests are available.

1 Total bacterial counts are made by cultivation of bacteria on membrane filters using an enriched all-purpose culture medium.

2 Tests for bacterial indicators of pollution.

a Coliform tests

1) The direct membrane filter tests for coliforms is one in which, after sample filtration, the membrane filter is incubated in contact with one or more special media. At least one of the media is a selective, differential medium including components which permit coliform bacteria to develop colonies easily recognizable by form, color, sheen, or other characteristics.

2) A verified membrane filter coliform test can be used when needed as a supplement to the direct membrane filter test. Pure cultures are obtained from individual colonies differentiated on the membrane filter and subjected to further cultural, biochemical, and staining tests to establish the identity of the colonies being studied.

3) The delayed membrane filter coliform test was developed to overcome bacterial changes frequently occurring when there is a delay of one to several days between sample collection and the initiation of laboratory tests. The test consists of sample filtration at or shortly after the time of sample collection. The inoculated filter is placed on a preservative medium and taken or sent to a laboratory, where it is transferred to a growth medium for the differentiation of coliform colonies. After incubation the culture is examined and the results are evaluated as for the direct membrane filter coliform test.

4) A medium and technique for detecting and counting fecal coliform bacteria has been developed and is called M-FC Broth. This medium currently is being used increasingly in water pollution studies.

b Selective, differential, culture media have been developed for direct cultural tests for members of the enterococcus group of bacteria.

3 Tests for pathogenic bacteria

a Workers are currently testing new media for the differentiation of members of the Salmonella-Shigella group of enteric pathogens. Available information indicates potential usefulness of a screening medium for differentiation of nonlactose-fermenting, non urease-producing bacteria.

b One medium has been used for screening tests in detection of Salmonella typhosa.

c Further confirmatory cultural, biochemical, and serological tests are necessary to establish the identity of bacteria differentiated with these screening media.

C Membrane filter techniques can be applied both in the laboratory and under field conditions. Several varieties of portable membrane filter field units have been developed on a commercial basis.

IV ADVANTAGES AND LIMITATIONS

This evaluation is limited to tests for the coliform group. Similar, but separate evaluations would have to be made for any other bacteriological test.

A Advantages

1 Results are obtained in approximately 24 hours, as compared with 48-96 hours required for the standard fermentation tube method.

2 Much larger, and hence more representative samples of water can be sampled routinely with membrane filters.

3 Numerical results from membrane filters have much greater precision (reproducibility) than is expected with the fermentation tube method.

4 The equipment and supplies required are not bulky. A great many samples can be examined with minimum requirements for laboratory space, equipment, and supplies.

B Limitations

1 Samples having high numbers of noncoliform bacteria capable of growing on Endo type culture media sometimes give difficulty. In such cases a high ratio of these noncoliform bacteria to coliforms results in poor sheen production, or even suppression, of the coliform organisms.

2 In samples having low coliform counts and relatively great amounts of suspended solids, bacterial growth sometimes develops in a continuous film on the membrane surface. In such cases the typical coliform sheen sometimes fails to develop.

3 Some samples containing as much as 1 milligram per liter of copper or zinc, or both, show irregular coliform bacterial results.

4 Occasional strains of bacteria growing on membrane filters producing sheen colonies prove, on subsequent testing, to be acid but not gas-producers from lactose. Where this occurs it may give a falsely-high indication of coliform density.

Such limitations as these are not frequent, but they do occur often enough to require consideration. In samples where these difficulties often occur, the best course of action often is to avoid use of membrane filter methods and use the multiple fermentation tube procedures.

V SUMMARY

The development of membrane filters and their bacterial applications has been discussed briefly, from their European origin to their current status in this country. Membrane filters currently available here have been described, and their properties have been considered. Applications of membrane filters in water bacteriology are indicated in general terms. Some of the advantages and limitations of membrane filter methods are presented for coliform tests.

REFERENCES

1 Goetz, Alexander. Materials, Techniques, and Testing Methods for the Sanitation (Bacterial Decontamination) of Small-Scale Water Supplies in the Field Used in Germany During and After the War. Technical Industrial Intelligence Branch. U.S. Department of Commerce. FIAT Final Report 1312. December 8, 1947.

2 Clark, Harold F., Geldreich, E.E., Jeter, H.L., and Kabler, P.W. The Membrane Filter in Sanitary Bacteriology. Public Health Reports. 66: 951-77. July 1951.

3 Goetz, A. and Tsuneishi, N. Application of Molecular Filter Membranes to the Bacteriological Analysis of Water. Journal American Water Works Association. 43:943-69. December 1951.

4 Clark, H.F., Kabler, P.W., and Geldreich, E.E. Advantages and Limitations of the Membrane Filter Procedure. Water and Sewage Works. 104: 385-387. 1957.

5 Public Health Service Publication 956. Public Health Service Drinking Water Standards. 1962.

6 Windle Taylor, E., and Burman, N.P. The Application of Membrane Filtratio Techniques to the Bacteriological Examination of Water. Journal Applied Bacteriology, 27:294-303. 1964.

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Descriptors: Filters, Membrane, Bacteria, Microorganisms, Laboratory Tests

MEMBRANE FILTER EQUIPMENT AND ITS PREPARATION FOR LABORATORY USE

I Some equipment and supplies used in the bacteriological examination of water with membrane filters are specific for the method. Other items are standard in most well-equipped bacteriological laboratories and are readily adapted to membrane filter work. This chapter describes needed equipment and methods for its preparation for laboratory use. Where more than one kind of item is available or acceptable for a given function, sufficient descriptive information is provided to aid the worker in selecting the one best suited to his own needs.

II EQUIPMENT FOR SAMPLE FILTRATION AND INCUBATION

A Filter Holding Unit

1 The filter holding unit is a device for supporting the membrane filter and for holding the sample until it passes through the filter. During filtration the sample passes through a circular area, usually about 35 mm in diameter, in the center of the filter. The outer part of the filter disk is clamped between the two essential components of the filter holding unit. (See Plate 1)

a The lower element, called the filter base, or receptacle, supports the membrane filter on a plate about 50 mm in diameter. The central part of this plate is a porous disk to allow free passage of liquids. The outer part of the plate is a smooth nonporous surface. The lower element includes fittings for mounting the unit in a suction flask or other container suitable for filtration with vacuum.

b The upper element, usually called the funnel, holds the sample until it is drawn through the filter. Its lower portion is a flat ring that rests on the outer part of the membrane filter disk, directly over the nonporous part of the filter support plate.

c The assembled filter holding unit is joined by a locking ring or by one or more clamps.

2 Characteristics of filter holding units should include:

a The design of filter holding units should provide for filtration with vacuum.

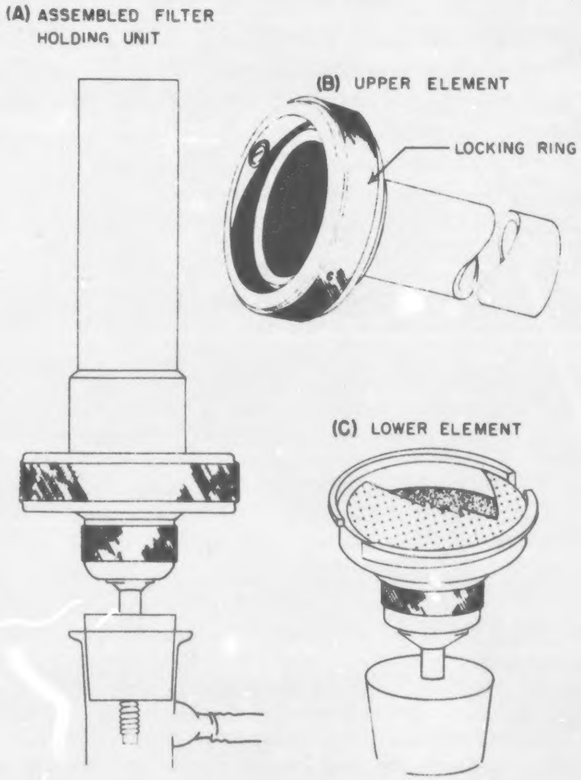


PLATE 1

NOTE: Mention of commercial products and manufacturers does not imply endorsement by the [illegible] and the Environmental Protection Agency.

b Filter holding units may be made of glass, porcelain, plastic, non-corrosive metal, or other impervious material.

c Filter holding units should be made of bacteriologically inert materials.

d All surfaces of the filter holding assembly in contact with the water sample prior to its passage through the membrane filter should be uniformly smooth and free from corrugations, seams, or other surface irregularities that could become lodging places for bacteria.

e Filter holding units should be easily sterilized by routine methods.

f The filter holding unit should be easily and quickly assembled and disassembled in routine operational use.

g Filter holding units should be durable and inexpensive. Maintenance should be simple.

3 Several forms of filter holding units have been developed for use with aqueous suspensions.

a SS 47 Membrane Filter Holder (Plate 2, Figure 1)

Conical-shape funnel with a 500 ml capacity. The base section includes a wirescreen membrane support. Funnel and base section are evenly joined by a locking ring mechanism. This assembly is designed to hold a 47 mm diameter membrane firmly in place allowing an effective filter area of approximately 9.6 square centimeters. The entire filter unit is made of stainless steel with the funnel interior having a mirror-like finish.

b The Millipore Pyrex Filter Holder (Plate 2, Figure 2)

The unit is made of pyrex glass with coarse grade fitted support in base for filter. The upper element of early models of glass filter holders had a capacity of 1 liter. Currently available units are supplied with upper elements having 300 ml capacity. The assembled filter holder is joined with a spring clamp which engages on flat surfaces encircling the upper and lower elements.

c Millipore Standard Hydrosol Filter Holder (Plate 2, Figure 3)

Most components of this unit are made of stainless metal. The porous membrane support plate is fine-mesh stainless steel screening. The upper element is a straight-sided cylinder 4 to 5 inches in diameter, constricted to a narrow cylinder at the bottom, to fit the lower element. Capacity of the funnel element is about 1 liter. The assembled filter holding unit is joined by a bayonet joint and locking ring. Accessories may be obtained for collection of small amounts of filtrate and for anhydrous sterilization of the filter holding assembly.

d Gelman "Parabella Vacuum Funnel" (Plate 2, Figure 5)

The unit is made of spun stainless steel. The locking ring is a bayonet-type fitting, and is spring-loaded. The funnel element has a 1-liter capacity.

e The Sabro Membrane Filter Holder (Plate 2, Figure 4)

The unit is mostly of stainless steel construction. The lower element is a combination vacuum chamber, filtrate receiver, and filter supporting element. It consists of a stainless steel cup with a metal cover. The cover is fitted with a rubber gasket permitting airtight fit of the cover into the top of the cup. A porous sintered stainless steel membrane support disk is mounted in the center of the cover. At the side of the beaker is a valve to which a pumping device can be fitted. The upper element is a stainless steel funnel with about 500 ml capacity. The assembled filter holding unit is joined by a locking ring at the base of the upper element. This engages on three spring clamps on the covering plate of the lower element.

f Millipore "Sterifil" filter unit (Plate 2, Figure 6)

A funnel and flask unit of polycarbonate with filter base and support of polypropylene. Manufacturers tables should be referred to regarding chemicals which may be present in the sample and their effect on the holder and flask elements. This unit can be safely sterilized under steam pressure.

FILTER HOLDING UNITS FOR AQUEOUS SUSPENSIONS

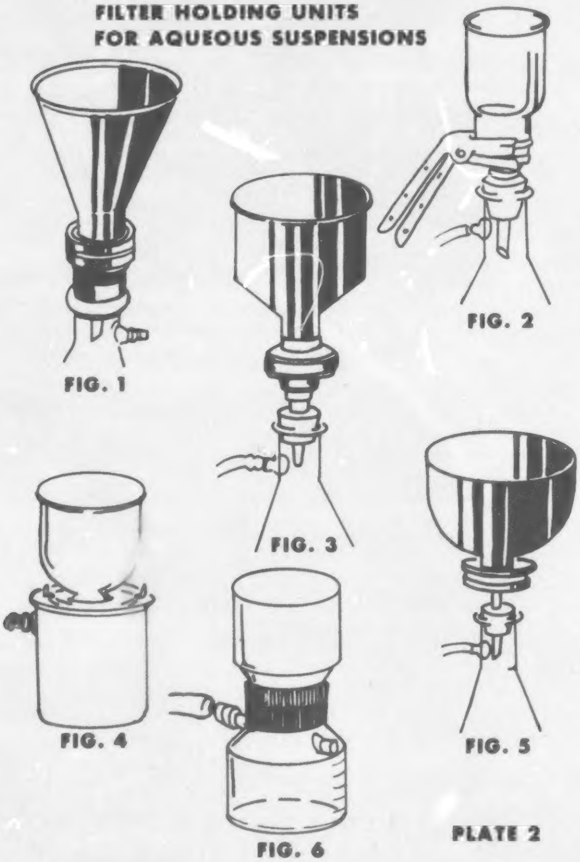


FIG. 1 FIG. 2 FIG. 3 FIG. 4 FIG. 5 FIG. 6

PLATE 2

4 Care and maintenance of filter holding units

a Filter holding units should be kept clean and free of accumulated foreign deposits.

b Metal filter holding units should be protected from scratches or other physical damage which could result in formation of surface irregularities. The surfaces in contact with membrane filters should receive particular care to avoid formation of shreds of metal or other irregularities which could cause physical damage to the extremely delicate filters.

c Some filter holding units have rubber components. The rubber parts may in time become worn, hardened, or cracked, necessitating replacement of the rubber part involved.

d The locking rings used in some kinds of filter holders have two or more small wheels or rollers which engage on parts of the filter holding assembly. Occasional adjustment or cleaning is necessary to insure that the wheels turn freely and function properly. On some units, the wheels are plastic, and are not intended to turn. When worn flat, they should be loosened, turned a partial turn, and tightened again.

B Membrane Filters and Absorbent Pads

1 The desired properties of membrane filters have been discussed elsewhere. Typical examples, commercially available include:

a Millipore Filters, Type HA, white, grid-marked, 47 mm in diameter

b S & S Type B-9, white, black-grid mark, 47 mm diameter c Oxoid cellulose acetate membrane filters, 4.7 cm, grid-marked

2 An absorbent pad for nutrient is a paper filter disk, usually the same diameter as the membrane filter. Absorbent pads must be free of soluble chemical substances which could interfere with bacterial growth. They should be of such thickness that they will retain 1.8 - 2.2 ml of liquid culture medium. During incubation of cultures on membrane filters an absorbent pad saturated with liquid culture medium is the substrate for each filter. Absorbent pads are supplied with the purchase of membrane filters. Additional absorbent pads may be purchased separately. Sterilization in an autoclave is recommended for absorbent pads.

C Vacuum

Water can be filtered through a membrane filter by gravity alone, but the filtration rate would be too slow to be practical. For routine laboratory practice, two convenient methods are available for obtaining vacuum to hasten sample filtration.

1 An electric vacuum pump may be used connected to a filtration apparatus mounted in a suction flask. The pump need not be a high-efficiency type. For protection of the pump, a water trap should be included in the system, between the filtration apparatus and the vacuum pump.

2 A water pump, the so-called "aspirator" gives a satisfactory vacuum, provided there is reasonably high water pressure.

3 In emergency, a rubber suction bulb, a hand pump, or a syringe, may be used for vacuum. It will be necessary to include some form of valve system to prevent return flow of air.

D Culture Containers (Plate 3)

Most membrane filter cultures are incubated in individual containers. Almost any form of culture container is acceptable if it is made of impervious bacteriologically inert material. The culture container should, or course, be large enough to permit the membrane filters to lie perfectly flat. The following are widely used:

1 Glass petri dishes

Conventional borosilicate glass culture dishes are widely used in laboratory applications of membrane filters. For routine work, 60 mm × 15 mm petri dishes are recommended. The common 100 mm × 15 mm petri dishes are acceptable, but are subject to difficulties.

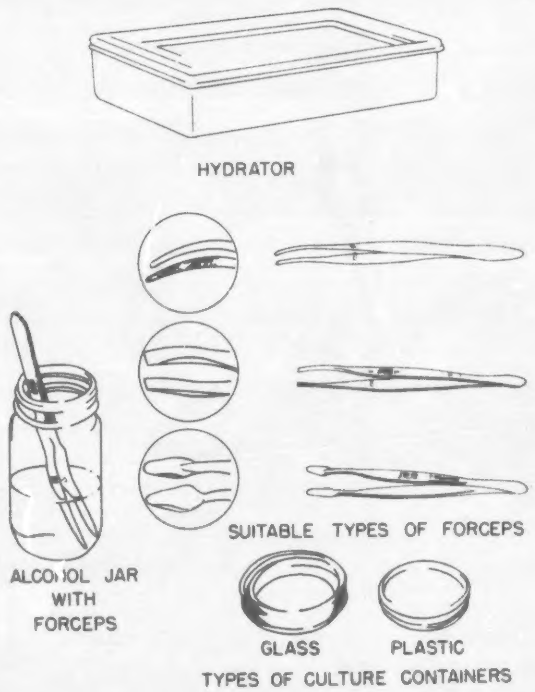


PLATE 3

2 Plastic petri dishes

Plastic containers have been developed for use with membrane filter cultures. Their cost is fairly low, and single-service use feasible. They cannot be heat-sterilized, but are supplied sterile. They must be free from soluble toxic substances. They can either be loose-fitting or of a tight lid to base friction fit.

E Other Equipment and Supplies Associated with Sample Filtration

1 Suction flask (Plate 4)

a Most types of filter holding apparatus are fitted in a conventional suction flask for sample filtration. While other sizes may be used, the 1-liter size is most satisfactory.

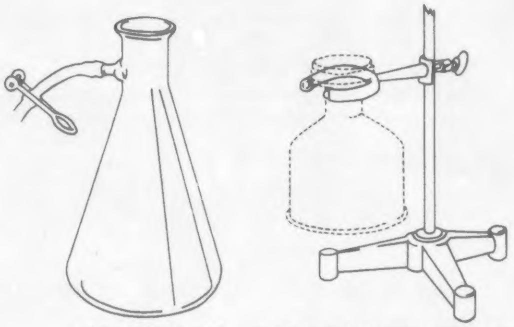
b The suction flask can be connected to the vacuum facility with thick-walled rubber tubing. Latex rubber tubing, 3/16" inside diameter, with wall thickness 3/32", is suggested. This tubing does not collapse under vacuum, yet it is readily closed with a pinch clamp.

c A pinch clamp on the rubber tubing is a convenient means of cutting off the vacuum from the suction flask during intervals when samples are not actually being filtered. It is most convenient to have the vacuum facility in continuous operation during sample filtration work.

d In laboratories conducting a high volume of filtration work, the suction flask may be dispensed. Filter-holding manifolds are available to receive up to three filtration units. The filtrate water is collected in a trap (in series with the vacuum source) which is periodically emptied.

e Another arrangement can be made for dispensing with the suction flask. In this case, the receptacle element of the filtration unit is mounted in the bench top. Instead of using a suction flask, the lower element of the filter holding unit has a dual connection with the vacuum source and with the laboratory drain. A solenoid-operated valve is used to determine whether the vacuum system or the drain line is in series with the filtration unit.

2 Ring stand with split ring (Optional) (See Plate 4)



SUCTION FLASK RING STAND WITH SPLIT RING

PLATE 4

When the filter holding unit is disassembled after sample filtration, the worker's hands must be free to manipulate the membrane filter. Upon disassembly of the filter holding unit, many workers place the funnel element, inverted, on the laboratory bench. Some workers, to prevent bacterial contamination, prefer a rack or a support to keep the funnel element from any possible source of contamination. A split ring on a ring stand is a convenient rack for this purpose.

3 Graduated cylinders

In laboratory practice, 100 ml graduated borosilicate glass cylinders are satisfactory for measurement of samples greater than 20 ml.

4 Pipettes and cans

a Graduated Mohr pipettes are needed for many procedures, such as measurement of small samples, and for preparing and dispensing culture media. Pipettes should be available in 1 ml and 10 ml sizes.

b Holding cans may be round or square but must not be made of copper. Aluminum or stainless steel are acceptable.

5 Alcohol jar with forceps (Plate 3)

a All manipulation of membrane filters is with sterile forceps. For sterilization, forceps are kept with their tips immersed in ethanol or methanol. When forceps are to be used, they are removed from the container and the alcohol is burned off.

b Forceps may be straight or curved. They should be designed to permit easy handling of filters without damage. Some forceps have corrugations on their gripping tips. It is recommended that such corrugations be filed off for membrane filter work.

6 A gas burner or alcohol burner is needed to ignite the alcohol prior to use of forceps.

7 Dilution water

The buffered distilled water described in "Standard Methods for the Examination of Water and Wastewater" for bacteriological examination of water is used in membrane filter methods. Dilution water is conveniently used in 99 ± 2 ml amounts stored in standard dilution bottles. Some workers prefer to use 9.0 ± 0.2 ml dilution blanks.

8 Culture medium

Bacteriological culture media used with membrane filter techniques are discussed at length in another part of this manual.

F Incubation Facilities

1 Requirements

a Temperature

For cultivation of a given kind of bacteria, the same temperature requirements apply with membrane filter methods as with any other method for cultivating the bacteria in question. For example, incubation temperature for coliform tests on membrane filters should be 35° C ± 0.5° C.

b Humidity

Membrane filter cultures must be incubated in an atmosphere maintained at or very near to 100% relative humidity. Failure to maintain high humidity during incubation results in growth failure, or at best, in small or poorly differentiated colonies.

2 The temperature and humidity requirements can be satisfied in any of several types of equipment.

a A conventional incubator may be used. With large walk-in incubators, it is extremely difficult to maintain satisfactory humidity. With most conventional incubators, membrane filter cultures can be incubated in tightly closed containers, such as plastic petri dishes. In such containers, required humidity conditions are established with evaporation of some of the culture medium. Because the volume of air in a tightly closed container is small, this results in negligible change in the culture medium. If glass petri dishes or other loosely fitting containers are used, the containers should be placed in a tightly closed container, with wet paper or cloth inside to obtain the required humidity conditions. A vegetable crisper, such as used in most home refrigerators,

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is useful for the purpose. (See Plate 3)

b A covered water bath maintaining 44.5°C ± 0.2°C is necessary for the fecal coliform test and this will necessitate the use of a water-bath having forced circulation of water.

III STERILIZATION OF MEMBRANE FILTER EQUIPMENT AND SUPPLIES

A Filter Holding Unit

1 When is sterilization necessary?

a The filter holding unit should be sterile at the beginning of each filtration series. A filtration series is considered to be interrupted if there is an interval of 30 minutes or longer between sample filtrations. After such interruption any further sample filtration is treated as a new filtration series and requires a sterile filter holding unit.

b It is not necessary to sterilize the filter holding unit between successive filtrations, or between successive samples, of a filtration series.

After each filtration the funnel walls are flushed with sterile water to free them of bacterial contamination. If properly done, the flushing procedure will remove bacteria remaining on the funnel walls and prevent contamination of later samples.

2 Methods for sterilization of filter holding unit

a Sterilization in the autoclave is preferred. Wrap the funnel and receptacle separately in Kraft paper and sterilize in the autoclave 15 minutes at 121°C. At the end of the 15 minutes holding period in the autoclave, release the steam pressure rapidly, to encourage drying of the filter holding unit.

b The unit may be sterilized by holding it 30 minutes in a flowing steam sterilizer.

c The unit may be immersed 2 to 10 minutes in boiling water. This method is recommended for emergency or field use.

d Some units (Millipore Stainless Unit) are available with accessories permitting anhydrous sterilization with formaldehyde. The method consists of introduction of methanol into a wick or porous plate in the sterilization accessory, assembly of the filter holding unit for formaldehyde sterilization, ignition of the methanol, and closure of the unit. The methanol is incompletely oxidized in the closed container, resulting in the generation of formaldehyde, which is bactericidal. The filter holding unit is kept closed for at least 15 minutes before use.

e Ultraviolet lamp sterilizers are convenient to use. A device now commercially available for ultraviolet sterilization of membrane filter funnel units.

B Sterilization of Membrane Filters and Absorbent Pads

1 Membrane filters

a Membranes are supplied in units of 10 in kraft envelopes, or in packages of 100 membranes. They may be sterilized conveniently in the packets of 10, but should be repackaged if supplied in units of 100. Large packages of filters can be distributed in standard 100 mm × 15 mm petri dishes, or they can be wrapped in kraft paper packets for sterilization.

b Sterilization in the autoclave is preferred. Ten minutes at 121° C or, preferably, at 116° C is recommended. After sterilization the steam pressure is released as rapidly as possible, and the filters are removed from the autoclave and dried at room temperature. Avoid excessive exposure to steam.

c In emergency, membrane filters may be sterilized by immersion in boiling distilled water for 10 minutes. The filters should first be separated from absorbent pads and paper separators which usually are included in the package. The boiling water method is not recommended for general practice, as the membranes tend to adhere to each other and must be separated from one another with forceps.

2 Absorbent pads for nutrient

a Unsterile absorbent pads can be wrapped in kraft paper or stacked loosely in petri dishes, and autoclaved with membrane filters (ten minutes or longer at 121° C or 116° C).

b After sterilization absorbent pads for nutrient should be dried before use.

C Glassware

1 Sterilization at 170° C for not less than 1 hour is preferred for most glassware (pipettes, graduated cylinders, glass petri dishes). Pipettes can be sterilized in aluminum or stainless steel cans, or they may be wrapped individually in paper. The opening of graduated cylinders should be covered with paper or metal foil prior to sterilization. Glassware with rubber fittings must not be sterilized at 170° C, as the rubber will be damaged.

2 Sterilization in the autoclave, 15 minutes at 121° C, is satisfactory, and preferred by many workers. When sterilizing pipettes it is important to exhaust the steam pressure rapidly and vent the containers momentarily. This allows the vapor to leave the can and prevents wet pipettes.

D Culture Containers

1 Glass petri dishes

a Petri dishes may be sterilized in aluminum or stainless steel cans, or wrapped in kraft paper or metal foil. They can be wrapped individually or, more conveniently, in rolls of up to 10 dishes.

b Preferably, sterilize glass petri dishes at 170° C for at least 1 hour.

c Alternately, they may be sterilized in the autoclave, 15 minutes at 121° C. After sterilization steam pressure should be released rapidly to facilitate drying of the dishes. Other suggested methods for sterilization of plastic dishes include exposure to ethylene oxide vapor (0.5 ml ethylene oxide per liter of container volume), or exposure to ultraviolet light. Ethylene oxide is a dangerous chemical being both toxic and explosive, and it should be used only when more convenient and safer methods are not available.

2 Plastic culture containers

a Because of the thermo-labile characteristics of the plastic, these containers cannot be heat sterilized. Manufacturers supply these in a sterile condition.

b For practical purposes, plastic dishes may be sterilized by immersion in a 70% solution of ethanol in water, for at least 30 minutes. Dishes must be allowed to drain and dry before use, as ethanol will influence the performance of culture media.

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Descriptors: Laboratory Facilities, Bacteriology, Microbiology, Enteric Bacteria, Filters, Membrane

MEMBRANE FILTER EQUIPMENT FOR FIELD USE

I INTRODUCTION

One of the most troublesome problems in bacterial water analysis is the occurrence of changes in the bacterial flora of water samples between the time of sample collection and the time the actual bacterial analysis is started. Numerous studies have been made on this problem. From these have come such recommendations (Standard Methods, 10th ed) as holding the sample at 0-10°C and starting laboratory tests as soon as possible after collection of the sample. Recommendations of Standard Methods, 12th Edition, was to hold the sample as close as possible to the temperature of the source and to start the laboratory tests preferably within 1 hour and always within a maximum of 30 hours after collection. Changes in the 13th edition of Standard Methods (1971) will again call for the icing of samples, and further, that samples of environmental waters be held for not more than 8 hours total elapsed time before samples are plated or used for microbiological testing. The 30 hour maximum elapsed sample holding time will still be retained for potable water samples.

A They would be useful in certain routine water quality control operations. Examples include such places as on board ships; some airlines, particularly in overseas operations; and some national parks. In each example, it is seen that there is an obvious difficulty in getting water samples to the examining laboratory in time for early examination.

B In addition, such units would be invaluable in emergencies when existing laboratories are overburdened or inoperative. Portable kits already have proven extremely helpful in testing many small water supplies in a short period of time. Further there is a predictable need for such equipment in the event of a wartime civil defense disaster. Experience of the Germans in the vicinity of Hamburg during World War II lends support to this concept.

The purpose of this discussion is to introduce some of the portable equipment which has been developed and to point out noteworthy features of each. Actual practice and experience with these units reveal strong point and weaknesses in each type.

The membrane filter method has been accepted by the Federal Government for the bacteriological examination of water under its jurisdiction. This acceptance was based on methods developed and procedures applied in fixed laboratories. While the use of field kits is not excluded, no special concession has been made regarding the standards of performance of membrane filter field kits. Thus, in planning to use a membrane filter field kit for the bacteriological examination of water, it is the responsibility of the individual laboratory to establish beyond reasonable doubt, by comparison with Standard Methods fermentation tube tests or established laboratory membrane filter methods, the value of use of the membrane filter field kit in determining the sanitary quality of water supplies examined.

II TYPES OF COMMERCIALLY AVAILABLE MEMBRANE FILTER EQUIPMENT FOR FIELD USE

A Sabro Water Laboratory

This unit represents a fixed membrane filter laboratory in miniature, with adaptations for special situations to be encountered in the field. Notable features:

1 The funnel unit supplied on older units is glass. A newer model has been released with an all-metal funnel unit.

2 The vacuum source is a hand pump (modified bicycle pump) or, optionally, an all-metal syringe.

3 The manufacturer sells prepared ampouled medium, in a liquid state. The medium should be kept at a cool

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NOTE: Mention of commercial products and manufacturers does not imply endorsement by the Federal Water Pollution Control Administration temperature, out of the light. Its useful shelf life is uncertain, but limited tests by this agency indicate that the medium performs acceptably with storage up to one year.

4 Incubation of the cultures is in an incubator drawer having a capacity of 18 1-ounce culture containers, or 36 plastic containers, and operates electrically at 110V, and with suitable converters, at 6V, 12V. A battery also can be used with this unit.

5 Sterilization of the funnel unit is carried out by a "light flaming technique" or, optionally, by immersion of the funnel unit in hot or boiling water.

6 Useful accessories provided include thermometer, alcohol lamp, measuring cup, and forceps.

B Millipore Field Monitor Units

These units differ radically from any other field equipment that has appeared. Significant reductions in bulk of equipment have been brought about through major changes in function and design of the usual equipment. Although not recommended for valid data due to differences in quantitation when compared to standard test procedures, the unit is useful for rapid field testing to establish "ball park" fiqures for later testing with approved test procedures. Notable features:

1 The funnel unit has been eliminated in its usual form. This has been done by development of a carefully fitted, single-use combination filtration unit and culture container. This feature eliminates most of the handling and use of accessory equipment.

2 The vacuum source is an all metal syringe with a fitting providing for direct connection to the culture container.

3 The culture medium provided by the manufacturer includes M-Endo Broth, MF, ready-to-use, in glass ampoules. These ampoules are so designed as to permit easy introduction of the culture medium into the culture container. Alternately, the manufacturer makes available a delayed-incubation medium in the ampoules. Other culture media can be used at the discretion of the user, but some difficulty can be anticipated in introducing the medium without special equipment.

4 Incubation of the cultures is provided in the field through use of an associated portable incubator and equipment carrying kit. This incubator has room for about 25 cultures. It is electrically operated, and through selection of available switching positions, operates at 6V, 12V, 110V, or at 220V.

5 Sterilization of components in the field is unnecessary. The culture containers and plastic tubes are single-use units supplied in a sterile condition. Samples do not come in contact with the syringe until after they have passed through the filter.

C Millipore Field Unit for Military Use

1 A modified Millipore field unit based on the case and incubator described in B, 4 above, has been adopted by the U.S. Department of Defense. This unit includes a miniaturized stainless metal funnel unit instead of the Monitors.

2 The vacuum source is an all-metal syringe.

3 Sterilization of funnel unit is by formaldehyde generated through incomplete combustion of methyl alcohol.

III COMMON DIFFICULTIES ENCOUNTERED IN COMMERCIALLY AVAILABLE FIELD EQUIPMENT

A The most conspicuous problem arising with field use of most units is their ultimate reliance on a fixed laboratory for essential supplies.

B These portable laboratories will permit simultaneous incubation of up to 30 membrane filters.

C For protracted field work, a fairly large amount of reserve supplies and equipment will be necessary. Such a reserve would include culture media, membrane filters, culture containers, fuel, and other expendable supplies required in the field, organized in a supplementary carrying case.

D No currently available field unit provides illumination or optical assistance for interpretation of results.

E Some of the sterilization methods recommended by manufacturers are unacceptable. If field sterilization in boiling water is needed, then there must be a heat source and a metal can or beaker. Such equipment could be carried in the case suggested in C above.

IV IMPROVISED FIELD EQUIPMENT

The initial cost of most of the commercially manufactured units has met some objection. This factor, coupled with need for additional accessory supplies and equipment, has aroused interest in improvised units. Such a unit could consist largely of equipment normally used in a fixed laboratory, packaged in one or two fiberboard cases.

A The funnel unit could be one of the familiar stainless steel units used in many laboratories; or it could be specially designed, smaller than ordinarily used, permitting use of up to a dozen or more upper filter holding elements in the field.

B The vacuum source could be the modified bicycle pump (leathers reversed), and provided with a by-pass valve. The suction flask could be the standard side-arm glass flask, or a metal unit could be devised.

C M-Endo Broth or LES Endo agar are suitable media. Both are available as dehydrated medium which must be reconstituted and boiled in the field. M-Endo Broth MF is now available in liquid form, sterile, in sealed ampules. A shelf life of approximately one year is stated when stored under moderate temperatures in the dark.

LES MF Holding Medium - Coliform requires merely dissolving in distilled water. No heating is necessary. Such medium would be an advantage where applicable.

D Sterilization of funnel units, graduated cylinders, media, etc., would be through immersion in boiling water for 2 minutes or longer, as indicated for the material being sterilized. Provision for boiling water is easy through use of a small camp stove or other simple burner.

Improvised equipment, such as discussed above, would have great usefulness in emergencies, where commercially available membrane filter field units are not on hand.

V In a separate outline are detailed descriptions of procedures for use of commercially available membrane filter field equipment. In some cases the suggested methods are different from those recommended by the manufacturers. In each case such departures are based on a series of experimental studies made by this agency, which suggested need for modification of existing recommendations.

REFERENCE

1 Laubusch, E. J. What You Should Know About the Membrane Filter, Public Works, 89: 106-13, 162-68, 1958.

This outline was prepared by H. L. Jeter, Chief, Program Support Training Branch, USEPA, Cincinnati, Ohio 45268

Descriptors: On-Site Laboratories, Field Investigations, Filters, membrane, Bacteria, Microorganisms, Laboratory Tests

PRINCIPLES OF CULTURE MEDIA FOR USE WITH MEMBRANE FILTERS

I INTRODUCTION

A Many kinds of membrane filter media have been described for use in bacteriological tests on water. This is noteworthy in view of the relatively few years the filters have been widely available in this country. This discussion is to consider several of these media in terms of their purposes, composition, and the ways in which they are used.

B Basic Considerations

1 Filtration of water sample through a membrane filter results in deposition of bacteria and particles of suspended matter on the filter surface. The bacteria can be cultivated in place if suitable culture medium is made available for their growth.

2 The bacteria are cultivated by placing the membrane on a pad of absorbent paper saturated with liquid culture medium, or on an agar medium. The culture medium diffuses through the pores of the filter, and is available to the bacteria on the opposite surface. Proper time, temperature, and humidity of incubation results in development of bacterial colonies. In principle, each bacterial cell multiplies to become a single bacterial colony.

3 Some culture media, satisfactory for tube cultures or agar plate cultures, do not perform well when used with membrane filters due to a selective adsorptive property of the filter itself. In the process of diffusion through the pores some components of the culture medium may be removed completely, or reduced in concentration. Thus, the composition of a given culture medium at the filter surface where it is available for bacterial growth may be different from its composition beneath the membrane filter.

There is evidence that improved cultural results sometimes are obtained with increased concentration of certain nutritive constituents of membrane filter culture media.

4 Pure cultures may be recovered from membrane filters and subjected to supplementary biochemical, cultural, and serological procedures for identification studies or for verification of interpretations based on direct observation of membrane filter cultures.

The same use can be made of agar plating media; however the membrane filter offers advantages due to the ability to concentrate organisms from a large volume of sample in which the organisms are present in low density.

C Applications of Membrane Filter Culture Media

The composition of bacteriological culture media designed for tube or plate cultures should be subjected to critical study before they are applied to membrane filter procedures. Media based on well-known bacteriological media have been modified for use with membrane filters for the following purposes in testing water.

1 Bacterial plate counts

2 Media for bacterial indicators of pollution

   a Coliform organisms

   b Fecal streptococcus group

   c Clostridium perfringens

3 Salmonella and other enteric bacterial pathogens

NOTE: Mention of commercial products and manufacturers does not imply endorsement by the FWPCA and the U.S. Department of the Inte

D Constituents of Membrane Filter Culture Media

Membrane filter media for the differentiation and counting of special groups of bacteria are based on the same principles used in differential agar plate media. Thus the components of a differential medium for membrane filter cultures include:

1 Substances favoring growth of the organisms for which the medium is designed. Inclusion of special peptones, fermentable carbohydrates, yeast or meat extracts, water and chemicals to adjust pH to a desired level are common methods of favoring growth of desired organisms.

2 Differential indicator system. The purpose of the indicator system is to produce characteristic colonies of the desired bacterial groups for easy recognition when present in a mixture with extraneous types of colonies. This is done through inclusion of (a), a component which is chemically changed by the organisms to be differentiated, and (b), indicator substances, which give visible evidence of an intermediate or end product resulting from a chemical change of substance (a).

3 Selective inhibitors. Some bacterial groups to be tested may be overwhelmingly outnumbered by extraneous types of bacteria. In such cases, it is necessary that substances be included in the medium which (a), prevent growth of a maximum number of kinds of extraneous bacteria, and (b), have minimum adverse affect on growth of the kind of bacteria for which the medium is designed.

E Variety of Methods of Using Media Available with Membrane Filter Methods

1 Single-stage tests

After sample filtration, the membrane filter is placed on a designated culture medium, and left there throughout the incubation period. The culture results are examined and interpreted directly.

2 Multi-stage tests

A membrane filter can be transferred from one culture medium to another without disturbance of bacteria or colonies on the filter. This is unique with membrane filter methods, and lends itself to a variety of cultural and testing procedures.

a The membrane filter, after sample filtration, can be incubated for a specified time on one medium, then transfered to a second medium. The method permits initiation of growth on enrichment medium, after which the membrane filter can be transferred to a less productive medium. With growth already begun, some differential culture media give better quantitative production than would be the case without preliminary incubation.

b After incubation on one or more media, colonies on the membrane filter can be subjected to biochemical tests with reagents too toxic to include in the culture medium. Such reagents may be flooded over the growth on the filter, or the filter may be placed on an absorbent pad saturated with the reagent, in order to make such tests.

c A third type of multi-stage test is one in which the membrane filter, after sample filtration, is placed temporarily on a medium containing a bacteriostatic agent. In the presence of such a substance, bacterial growth is inhibited or slowed greatly, but the organisms are not killed. During a limited period, the membrane filters may be transported or stored at ambient temperatures. The filter can be transferred later to a suitable medium and incubated for development of colonies.

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II CULTURE MEDIA FOR TOTAL BACTERIAL COUNTS ON MEMBRANE FILTERS

A Concepts

1 Strictly, a "total" bacterial count medium is nonexistent. No single medium and incubation procedure can provide simultaneously the full range of oxygen requirements, needs for special growth substances, pH requirements, etc., of all the kinds of bacteria found in water.

2 Actually, "total" bacterial counts are counts of the bacteria developing visible colonies on a defined culture medium at a known pH after incubation for a set time and temperature under aerobic conditions.

3 Within the foregoing limitations, the following criteria offer a useful basis for selection of membrane filter media to be used for estimates of the bacterial density in water.

a The medium and its method of use should produce a maximum number of colonies from all types of water. The colony yield should compare favorably with the bacterial counts determined as described in "Standard Methods for the Examination of Water, Sewage and Industrial Wastes." 13th Ed. (1971).

b The colonies should develop rapidly to a sufficient size to be counted after a minimum incubation period. At present, best results with membrane filter methods are obtained after about 18 hours incubation.

c The medium should be one which is reproducible and routinely available in laboratories.

B Composition of Total Count Media for Membrane Filters

Almost any rich, general growth promoting culture medium is acceptable for total bacterial counts on membrane filters. Several such media have been suggested especially for membrane filter methods. These differ only in minor aspects, and can be discussed as a group. For details of composition and specific applications of each, see the media formulations elsewhere in this manual.

1 Growth promoting substances: All the substances included in these media are included to encourage growth of a maximum number of kinds of bacteria. Most workers agree that the peptone should be used in twice the concentration usually found in conventional tube or agar plating media.

2 Indicator substances are unnecessary with total count media.

3 Substances for the selective inhibition of certain bacterial groups are not included in total count media.

C Problems Encountered with Total Count Media

Bacterial colonial growth habits on membrane filters are similar to their surface growth habits on similar agar plate media.

1 As with agar plate media, some species of bacteria grow continuously, spreading over the surface of a membrane filter, tending to obscure nonspreading colonies which otherwise could be counted.

2 Some samples contain an appreciable amount of particulate matter. In sample filtration, this is deposited on the surface of the membrane filter with the bacteria. When the culture medium diffuses through the filter, a capillary film of liquid culture medium accumulates around the particles of extraneous matter. Bacteria not ordinarily considered "spreaders" sometimes develop confluent colonies due to the film of liquid medium accumulating around such particles.

D What is the best total count medium for use with membrane filters?

Because of the relative ease of preparation, most workers prefer the commercially prepared dehydrated media. Difco M-Enrichment Broth (B 408) or Baltimore Biological Laboratories' M-Enrichment Broth (No. 331) are used interchangeably. Total colony productivity of these media is equivalent to that of media prepared from the individual components.

III CULTURE MEDIA FOR TOTAL COLIFORM TESTS ON MEMBRANE FILTERS

A Concepts

The nature of membrane filter culture methods imposes a different definition of coliform bacteria than the Standard Methods definition.

1 Standard Methods fermentation tube method. "The coliform group includes all of the aerobic and facultative anaerobic Gram-negative nonsporeforming rod-shaped bacteria which ferment lactose with gas formation within 48 hours at 35°C."

2 Membrane Filter Methods: "In the membrane filter procedure, all organisms that produce a colony with a metallic sheen in 22-24 hours are considered members of the coliform group. The sheen may appear as a small central focus or cover the entire colony." The guiding principle is that any amount of sheen is considered positive.

3 The Standard Methods definition of coliforms requires demonstration of the ability of organisms to produce gas through the fermentation of lactose. The membrane filter method does not lend itself to the demonstration of gas production. It relies instead on the development of a particular type of colony on an Endo type of culture medium. The culture medium is one in which lactose, basic fuchsin, and sodium sulfite comprise an indicator system to cause differentiation of coliform colonies. While the bacterial groups measured by membrane filter methods are not identical with the group measured by Standard Methods procedures, they are believed to be essentially the same, and to have equal sanitary significance.

B Composition of Coliform Media for Membrane Filters

Several different media have been suggested for coliform tests on membrane filters. The components of these media can be classed into three convenient groups for general considerations.

1 Growth-promoting substances. Growth of bacteria on all the media is favored by the inclusion of such components as peptones (as Neopeptone, Thiotone Casitone, Trypticase, and other proprietary peptones), yeast extract, dipotassium phosphate (for adjustment of reaction of the medium), and distilled water. Lactose is included in all these media. It serves doubly, to favor growth of coliform bacteria, and as an essential component of the systems for differentiating coliform colonies.

2 Two kinds of differential indicator systems are available for demonstration of lactose fermentation on membrane filters.

a Lactose-basic fuchsin-sodium sulfite system (Endo type media).

1) Media using this system include lactose and a suitable concentration of basic fuchsin which has been partially decolorized with sodium sulfite.

2) The basic fuchsin-sodium sulfite complex requires very careful standardization. An excess of either component results in an unsatisfactory culture medium.

3) The indicator system demonstrates lactose fermentation as follows:

a) The coliform bacteria produce aldehyde as an intermediate product of the fermentation of lactose.

b) The aldehyde is "complexed" by the sodium sulfite-basic fuchsin indicator. In this process a reaction occurs in which red color is restored to the basic fuchsin. Colonies of bacteria fermenting lactose assume the color of the restored fuchsin. As the restored dye accumulates, it apparently precipitates on the colony, giving the colony a characteristic green-gold surface sheen. The reaction occurs best in an alkaline medium. The culture medium is adjusted to pH 7.5.

4) Endo type media require very careful standardization for successful use in the laboratory. Most workers prefer to use a commercially prepared and standardized medium. M-Endo Broth MF is the recommended coliform medium for use with membrane filters.

b pH indicator system

1) Media using this system rely on detection of pH change due to the accumulation of organic acids, end products of lactose fermentation.

2) Bromcresol purple, for example, is a pH indicator, approaching yellow at more acid pH. Colonies fermenting lactose and accumulating organic acids therefore turn yellow.

3) Studies in England with membrane filters for coliform tests have been based on a modification of MacConkey's Medium, using this principle of colony differentiation in coliform tests.

3 Inhibitory substances in membrane filter coliform media

a Confusing and erroneous results in coliform detection can be caused by

1) the overgrowth of the membrane filter by extraneous nonlactose fermenting bacteria, preventing coliform colonies from developing the characteristic color and sheen; and

2) the development of sheen colonies of lactose fermenting bacteria which produce acid but not gas in the fermentation of lactose.

b These difficulties can be reduced through incorporating of substances harmless to coliform bacteria but which have inhibitory effect on growth of extraneous forms. Attention must be given to the concentration of such substances, as excessive amounts also will reduce the productivity of the medium for coliform colonies. The following components of various culture media have proven useful in suppressing growth of noncoliform bacteria on membrane filters.

1) Basic fuchsin-sodium sulfite. Although these compounds are included in Endo-type media for their role in differentiating coliforms from other types of colonies, they are effective in preventing the growth of many of the noncoliform bacteria occurring in water samples.

2) Ethanol (95%. . . NOT denatured) is included in M-Endo Broth MF. In the concentration used, ethanol suppresses growth of some kinds of noncoliform bacteria, and tends to limit the colony size of others. In addition, the ethanol seems to increase the solubility of some of the other components of the media.

3) Sodium desoxycholate or bile salts are used in such media as M-Endo Broth MF, and in the modified MacConkey's Medium for membrane filters used in British studies. They are included primarily for their inhibitory effect against Gram-positive cocci and spore formers.

C Methods Available for Using Coliform Media with Membrane Filters

1 Single-stage coliform tests

a After sample filtration, the membrane filter is incubated for the desired time on a selective coliform differentiating medium.

b The coliform colonies are counted without further tests.

c M-Endo Broth MF and LES Endo Agar Media are alternate standard single-stage coliform media.

2 Two-stage coliform tests

a Immediate coliform test

1) After sample filtration, the membrane filter is incubated $1\frac{1}{2}$ - 2 hours on the enrichment medium of lauryl tryptose broth.

2) The membrane is then transferred to a new absorbent pad saturated with the standard differential medium for coliform bacteria, and incubated for 20-22 hours at 35 ± 0.5C.

3) The coliform colonies are counted without further tests.

4) This test procedure, based on EHC Endo Medium, was described in the 10th edition of Standard Methods. With the 12th edition, an official two-stage coliform test has been adopted, based on LES Endo Agar Medium.

b Delayed incubation coliform test

1) After sample filtration, the membrane filter is placed on an absorbent pad saturated with benzoated Endo Medium or with LES Holding Medium. The filter may be preserved up to 72 hours at ambient temperatures. During this time it can be transported or stored. Growth is stopped or greatly reduced.

2) The membrane filter can be transferred to a fresh absorbent pad, saturated with such a medium as M-Endo Broth MF, or to LES Endo Agar and incubated up to 24 hours.

3) The differentiated coliform colonies are counted as with other membrane filter coliform media.

4) This test procedure makes it possible to filter samples in the field, place the filters on preservative medium, then mail or transport them to the laboratory for completion of the bacteriological examination. The procedure is designed to eliminate the need for maintaining sample temperature in the interval between sample collection and initiation of the bacteriological examination. In addition, the method should produce results more nearly reflecting the quality of the source water than is available with other methods of collecting and testing samples.

3 Verified membrane filter coliform test

a This is used to verify the interpretation of differentiated colonies on any type of membrane filter coliform medium. The test is suggested for: self-training of laboratory workers, for evaluation of new or experimental media, and in any water examination in which the interpretation of results is in doubt or likely to be involved in legal controversy.

b The test consists of obtaining pure cultures from differentiated coliform-like colonies on membrane filters, and subjected them to further cultural and biochemical tests to establish their identity as Gram-negative non-sporeforming bacilli which ferment lactose with gas production. The technical procedures are described elsewhere in this manual.

IV MEDIUM FOR THE FECAL COLIFORM TEST

A Concepts

The selective effect of elevated temperature has been the most important development in fecal coliform tests since 1904. In that year, Eijkman discovered that coliform bacteria from the gut of warm-blooded animals produced gas from glucose at 46°C, while the majority of coliform bacteria from other sources did not. Media variations were of only secondary importance.

Much medium variation has resulted from attempts to select for Escherichia coli only, as the fecal coliform. While E. coli is usually the predominant coliform in human (and animal) feces, other types are present, including the alleged soil and plant coliform, Aerobacter aerogenes in very large numbers. All coliforms demonstrated by isolation to have arisen in feces are called here fecal coliforms and are measured empirically by the fecal coliform tube test. Membrane filter tests reflect divergence of attitude on indicators of fecal origin. Delaney et al. (1962) have published: Measurement of E. coli Type I by the Membrane Filter. Geldreich et al. (1965) have presented: Fecal-Coliform-Organism Medium for the Membrane Filter Technique Temperatures are the same but media are different. Because the fecal coliform test appears more convenient, it will be emphasized.

B Composition of Fecal Coliform Medium MFC

1 MFC medium is a rich growth medium containing lactose, proteose peptone no. 3, tryptone and yeast extract. A level of .3% sodium chloride produces favorable osmotic balance. Vigorous growth results. A practical result is shortening of test time to 24 hours.

The growth constituents are similar to those of the tube test for fecal coliform. Both have 0.15% bile salts to select for coliforms but elevated temperature is the more important selective factor.

2 The indicator system of aniline blue results in blue fecal coliform colonies. Nonfecal coliform colonies, generally few, are gray to cream-colored.

C Special Problems with MFC Broth Medium

1 Temperature control must be accurate. Current recommendations call for 44.5 ± 0.2°C and the temperature to be maintained in a water incubator of forced circulation.

2 Temperature equilibration must be rapid. Nonfecal coliforms may initiate growth at lower temperatures and subsequently give false positive blue colonies when incubated at 44.5°C. No more than 20 minutes lapse of time is recommended from filtration to incubation. Submergence in waterproof plastic bags reduces actual temperature equilibration to 10 - 12 minutes.

3 Rosolic acid presents some problems in preparation. It is practically insoluble in water and of limited stability in alkaline solution. A 1% solution in 0.2 N NaOH should be prepared and this added to the medium as recommended by the manufacturer.

V MEDIA FOR FECAL STREPTOCOCCUS TESTS

A Introduction

1 The development of membrane filter culture media for the fecal streptococci reflects the continuing interest in this group of bacterial indicators of pollution. The productivity of enterococcus media recently has been greatly increased.

2 Standards of performance of a good fecal streptococcus medium correspond with those of a good coliform medium on membrane filters. Thus, the requirements of productivity, specificity, ease of use, and reproducibility of the medium, are equally applicable to medium for the detection and enumeration of the fecal streptococci.

B KF Agar

1 This streptococcus medium was developed at SEC by Kenner, et al. and designated KF agar.

While KF medium is productive for detection and enumeration of the Fecal Streptococcus Group, its use is hampered by nonspecificity. Studies have demonstrated that the medium supports growth of S. bovis, and other forms common in animals, but not numerous in the fecal excreta of humans.

2 Composition of KF Agar

a Nutritive requirements of the fecal streptococci are supplied by peptone, yeast extract, sodium glycerophosphate, maltose, lactose, and distilled water.

b The indicator system is phenol red and 2, 3, 5 triphenyl tetrazolium chloride. On KF Agar used with membrane filters, the fecal streptococcus colonies develop as small colonies, up to 2 mm in diameter, colored various shades from pale pink to a dark wine color.

c The selective component of KF Agar is sodium azide, used in 0.04% concentration.

REFERENCES

1 APHA, AWWA, FSIWA. Standard Methods for the Examination of Water and Wastewater. 12th Edition.

2 Clark, H.F., Geldreich, E.E., Jeter, H.L. and Kabler, P.W. The Membrane Filter in Sanitary Bacteriology. Public Health Reports. 66:951-77. 1951.

3 Fifield, C.W. and Schaufus, C.P. Improved Membrane Filter Medium for the Detection of Coliform Organisms. J. Amer. Water Works Assn. 50:2:19[illegible]-6. 1958.

4 Geldreich, E.E., Clark, H.F., Huff, C.B. and Best, L.C. Fecal-Coliform-Organism Medium for the Membrane Filter Technique. J. Amer. Water Works Assn. 57:2:209-14. 1956.

5 Kabler, P.W. and Clark, H.F. Use of Differential Media with the Membrane Filter. Am. Jour. Pub. Health. 42:390-92. 1952.

6 Kenner, Bernard A., Clark, H.F. and Kabler, P.W. Fecal Streptococci. I. Cultivation and Enumeration of Streptococci in Surface Waters. Applied Microbiology. 9:15-20. 1961.

7 McCarthy, J.A., Delaney, J.E. and Grasso, R.J. Measuring Coliforms in Water. Water and Sewage Works 108:238. 1961.

8 Rose, R.E. and Litsky, W. Enrichment Procedure for Use with the Membrane Filter for the Isolation and Enumeration of Fecal Streptococci in Waters Microbiol. 13:1:106-9. 1965.

9 Slanetz, L.W., Bent and Bartley. Use of the Membrane Filter Technique to Enumerate Enterococci in Water. Public Health Reports. 70:67. 1955.

10 Slanetz, L.W., Bartley, C.H. and Ray, V.A. Further Studies on Membrane Filter Procedures for the Determination of Numbers of Enterococci in Water and Sewage. Proc. Soc. Am. Bacteriologists. 56th General Meeting. 1956.

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Descriptors: Cultures, Bacteria, Enteric Bacteria, Coliforms, Fecal Coliforms, Streptococcus, Membrane Filters.

SELECTION OF SAMPLE FILTRATION VOLUMES FOR MEMBRANE FILTER METHODS

I INTRODUCTION

A Wide Range of Filtration Volumes

1 The membrane filter permits testing a wide range of sample volumes, from several hundred milliliters to as little as 0.0001 ml, or even less. Suitable dilution of sample volumes smaller than 1.0 ml may be required for accuracy of sample measurement.

2 While the method lends itself to a wide range of sample volumes, the filter has limitations in the number of isolated (or countable) differentiated colonies which can develop on the available surface area. Figure 1 illustrates a common pattern of colony counts over a wide range of sample filtration volumes.

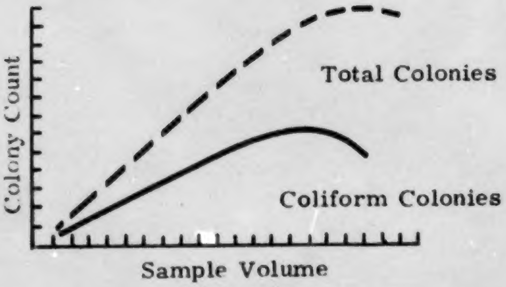

Figure 1

a The graph is based on coliform determinations using M Endo Broth MF. The line designated "Total Colonies" includes both coliform and noncoliform colonies. The "Coliform Colonies" line refers only to differentiated colonies having the typical color and sheen of coliform colonies on the medium

b For both the total colonies and the coliform colonies there is a proportional relationship between colony count and sample volume over much of the range of sample volumes. With increasing colony counts, there are some levels above which the proportional relationship fails, both for total and for coliform colonies.

c In the straight lines in Figure 1, where there is proportionality between colony counts and filtration volumes, it is possible to compute density of bacteria in the sample, based on the equation:

$$\begin{array}{l}\text{No. organisms}\\ \text{per 100 ml}\end{array} = 100 \times \frac{\text{No. colonies counted}}{\text{No. ml of sample filtered}}$$

The equation is not quantitatively reliable in the curved portions of the lines.

B Scope of this Presentation

1 To explain limitations on quantitatively reliable colony counts on membrane filters.

2 To present numbers of colonies acceptable for quantitative tests with availabl media.

3 To demonstrate the different quantitative bacterial density ranges determined by single-volume filtrations, with available media.

4 To demonstrate quantitative ranges covered by a series of filtration volum with currently-used differential media.

5 To provide guidance for selection of sample filtration volumes under the following practical conditions:

a When there is need to determine compliance with established bacteriological water quality standards.

b When there is need to determine density of a specified bacterial group.

1) In the absence of prior bacteriological data, and

2) When prior bacteriological data are available.

II LIMITATIONS ON COLONY COUNTS USED FOR QUANTITATIVE WORK

A Bacterial Density

1 The minimum sample volume should result in production of at least 20 colonies of the bacteria being counted. Sample volumes yielding lesser numbers of colonies are subject to unacceptably large random variations in the computed bacterial density, determined as above (I, A, 2).

2 The maximum acceptable colony density, for quantitative determinations, is variable with the bacterial group tested and the medium used. Factors influencing maximum acceptable colony density include:

a Size of colonies. In principle, each colony should represent one bacterial cell deposited on the filter, or, conversely, each bacterial cell deposited on the filter should result in production of a recognizable colony. Media producing relatively large colonies (as in the fecal coliform test) will support smaller numbers of colonies on the filter than media producing smaller colonies (such as fecal streptococci). If the colony size is large and the number of bacteria deposited on the filter is great, some colonies will represent two or more cells initially deposited on the filter, and the quantitative reliability of the test is impaired.

b Selectivity of medium. Highly selective media permit growth of relatively few colonies of extraneous, unwanted, bacteria. The available area of the filter is occupied primarily by colonies of the group tested. Thus, with a highly selective medium such as that used for fecal streptococci, it is reasonable to expect good quantitative results with relatively high colony counts. Conversely, media having limited selectivity (such as Endo-type media for coliforms) supports growth of considerable numbers of extraneous bacterial colonies, and it is necessary to place arbitrary limitations on the number of colonies per membrane in quantitative studies.

c Biochemical interference between neighboring colonies. Associated with the physical crowding effects noted in (b) above, sheen production of coliform colonies may be inhibited by overcrowding of colonies. This reinforces the need for restriction of colony density on the filter.

B Suspended Matter

1 Particulate matter in the sample can be a limitation in application of membrane filters, especially when the amount of suspended matter is relatively great and the bacterial density is low.

2 Difficulties from suspended matter in the sample may be apparent in several ways.

a The pores of the filter may be occluded, limiting the volume of sample that can be filtered. This problem has been noted in waters rich in clays and in waters containing large populations of certain diatoms or other algae.

b Fibrous matter can be troublesome, due to the tendency for a capillary film of liquid culture medium to form around the fibers. Colonies in contact with such fibers tend to grow along the path of the fibers, assuming highly irregular forms. Sometimes these colonies cover abnormally large areas of the filter surface.

c A more or less continuous mat of particles may be collected from some samples, with each particle soon surrounded by a film of liquid culture medium. On such filters, distinct colonies usually fail to develop as discrete entities, but grow in a more or less continuous film over the entire surface of the filter.

3 Problems due to particulate matter often can be reduced by filtration of the selected volume of water in two or more smaller increments, through separate filters. In effect, this is a means of enlarging the available surface area of the filter.

Prefiltration of the sample through a coarse filter for preliminary removal of extraneous particulate matter is not recommended in quantitative work. Prefiltration invariably results in removal of unpredictably large numbers of bacterial cells.

In some cases the problem of particulates cannot be solved, and it must then be conceded that the membrane filter method is not acceptable for such samples. It then becomes necessary to resort to other procedures, such as the dilution tube method or agar plating methods.

III LIMITS ON NUMBER OF COLONIES ON FILTERS WITH VARIOUS MEDIA

Referring to Figure 1, a specific number of colonies is not shown for acceptable proportionality between colony number and filtration volume. Fixed limits cannot be stated for all test situations, for these limits are somewhat variable from one culture medium to another and from one sample source to another.

The recommended limits shown in Table 1 are empirical values based on research experience. It is believed that quantitative determinations of acceptable statistical reliability can be obtained if the determinations are based on colony counts within the limitations shown.

IV RANGE OF BACTERIAL DENSITIES COVERED BY SINGLE-VOLUME FILTRATIONS

A The equation used in Section I of this outline can be used with any sample filtration volume to determine the bacterial density range over which acceptable counts can be made.

For example, assume that a sample of 10 ml is used for a quantitative determination of total coliforms. Based on Table 1, quantitative determinations should be based on a filtration volume yielding 20 - 80 coliform colonies. Compute the coliforms per 100 ml based on 20 colonies and on 80 colonies per filter. This will be the bacterial density range covered by a 10 ml filtration volume, thus:

for 20 colonies:

$$\text{No. coliforms per 100 ml} = 100 \times \frac{20}{10} = 200$$

and for 80 colonies,

$$\text{No. coliforms per 100 ml} = 100 \times \frac{80}{10} = 800$$

Thus, a 10 ml sample portion is appropriate for determination of total coliforms in the range 200 - 800 per 100 ml.

B Table 2 illustrates the ranges covered for several filtration volumes, with colony counts in the ranges 20 - 60, 20 - 80, and 20 - 100 per filtration volume.

Table 1. RECOMMENDED COLONY COUNT RANGES FOR QUANTITATIVE DETERMINATIONS WITH MEMBRANE FILTER TESTS

Test	No. colonies		Medium	Remarks
	Minimum	Maximum		
Total Coliform	20	80	M Endo Broth MF, LES Endo Medium	Not more than 200 colonies of all types
Fecal Coliform	20	60	M FC Broth	
Fecal Streptococci	20	100	M Enterococcus Agar, KF Agar	
Total Counts	20	200	M Enrichment Broth	Spreaders may require adjustment

Table 2. RANGES COVERED BY REPRESENTATIVE FILTRATION VOLUMES

Ml sample filtered	Bacterial count per 100 ml based on			
	20 colonies	60 colonies	80 colonies	100 colonies
100	20	60	80	100
10	200	600	800	1000
1	2000	6000	8000	10, 000
0. 1	20, 000	60, 000	80, 000	100, 000
0. 01	200, 000	600, 000	800, 000	1, 000, 000

C Application of a Series of Filtration Volumes

1 Examination of Table 2 shows that for quantitative work on membrane filters, to extend the range of any test, it is necessary to filter two or more different sample volumes. The worker uses the one sample volume yielding a quantitatively acceptable number of colonies to compute the bacterial count per 100 ml.

2 Further, it can be seen that varying the filtration volumes by decimal increments will be inappropriate; there are values within the total range covered in which the colony number would fall outside the critical counting range for the test being made.

3 In order to give maximum assurance that a series of varying filtration volumes will yield at least one membrane with an acceptable number of colonies, the range of filtration volumes should be along these lines:

a Total coliform counts should be based on filtration volumes varying by a factor of [illegible], or less.

b Fecal coliform counts should be based on filtration volumes varying by a factor of 3, or less.

c Fecal streptococcus counts should be based on filtration volumes varying by a factor of 5, or less.

V SELECTING FILTRATION VOLUMES FOR MEMBRANE FILTER TESTS

A Total Coliform Counts

1 Determination of compliance with existing bacterial quality standards.

a For all tests to determine whether water meets PHS Drinking Water quality standards, minimum sample sizes are prescribed as 50 ml, with 100 ml sample volumes suggested.

b With tests in which it is assumed that coliforms are present in some numbers, and the test is to determine whether some limiting standard (as 1000 per 100 ml in natural bathing waters, prescribed by some agencies), another approach is suggested. Here, select the sample filtration volume which would be quantitatively most acceptable to count coliforms at the limiting value. For example, with a limiting value of 1000 per 100 ml:

$$\text{No. organisms per 100 ml} = 100 \times \frac{\text{No. colonies counted}}{\text{No. ml of sample filtered}}$$

This previously given equation can be rearranged to:

$$\text{Sample filtration volume in ml} = 100 \times \frac{\text{No. colonies counted}}{\text{No. organisms/100 ml}}$$

and from this:

$$\text{Sample filtration volume in ml} = 100 \times \frac{50}{1000}$$

$$= 5$$

(The value 50 is the midrange number of colonies for an acceptable colony count of 20 - 80 for computing coliforms per 100 ml)

2 In quantitative work, to determine number of coliforms per 100 ml the worker may or may not have prior information or standards to use as guidance in selecting filtration volumes.

a In absence of prior bacteriological data

1) Unpolluted raw surface water, 1, 4, 15, and 60 ml samples will cover a count range of 33 - 8000 per 100 ml.

2) Polluted raw surface water, 0.02, 0.08, 0.15, and 0.5 ml samples will cover a count range of 4000 to 400,000 per 100 ml.

3) Sewage and dilute sewage, with filtration volumes of 0.0003, 0.001, 0.003, and 0.01, will cover a count range of 200,000 to 27,000,000 per 100 ml.

b If prior coliform data are available

Use the equation:

$$\text{Basic filtration volume in ml} = 100 \times \frac{50}{\text{Average coliform count}}$$

Example: Assume that prior data indicate average coliform count of 35,000 per 100 ml. Using the equation:

$$\text{Basic filtration volume in ml} = 100 \times \frac{50}{35,000}$$

$$= 0.143 \text{ ml}$$

Round off the filtration volume to 0.15 ml.

To assure a reasonable count-range, filter increments of 0.04 and 0.60 ml in addition. This will provide for acceptable coliform counts in the range of 3300 to 200,000 per 100 ml

B Fecal Coliform Counts

1 Currently, no drinking water standards are based on fecal coliform organisms.

Many states have environmental water quality standards which are based on fecal coliform organisms.

2 Determination of fecal coliforms in the absence of prior data.

a Unpolluted raw surface water: Filter 1, 3, 10, and 30 ml sample portions. These volumes will cover a fecal coliform range of 67 - 6000 per 100 ml.

b Polluted raw surface water: Filter portions of 0.1, 0.3, 1.0, and 3.0 ml. This will cover a fecal coliform count range of 670 to 60,000 per 100 ml.

c Sewage and dilute sewage: Filter sample portions of 0.0003, 0.001 and 0.003 ml. This will provide for counts of 670,000 to 20,000,000 per 100 ml.

3 Determination of fecal coliforms in presence of prior data

a When previous fecal coliform counts are available:

$$\text{Filtration volume in ml} = 100 \times \frac{40}{\text{Av. fecal coliform count per 100 ml}}$$

Example: Prior data show 8000 fecal coliforms per 100 ml.

$$\text{Basic filtration volume in ml} = 100 \times \frac{40}{8000} = 0.5$$

Filter volumes of 0.15, 0.5 and 1.5 ml. This will be suitable for fecal coliform counts over the range 1300 to 40,000.

b When previous total coliform data are available but no fecal coliform data are available, use the total coliform value as above, but filter 3x and 9x the computed basic volume.

Example (from above): Computed basic value = 0.5 ml

Filter volumes of 0.5, 1.5 and 5.0 ml.

C Fecal Streptococcus Determinations

1 In absence of prior data

a Unpolluted raw surface water: Filter sample portions of 1, 5, 25 and 100 ml. This will provide for fecal streptococcus counts in the range 20 to 10,000 per 100 ml.

b Polluted surface water: Filter sample portions of 0.1, 0.5, and 2.0 ml. This will provide for fecal streptococcus counts in the range 1000 to 100,000 per 100 ml. Provision for rather high counts of fecal streptococci is made because of possible situations in which pollution of the water originates from domestic or wild animals. In the event that such pollution is highly improbable, a filtration series of 0.2, 1.0, and 5.0 ml (covering a count range of 400 to 50,000 per 100 ml) would be more appropriate.

2 When prior data are available

a If coliform, but not fecal streptococcus data are available, compute a basic filtration volume as in A. 2 above, but use the average coliform count as a point of reference. If significant pollution from domestic or wild animals is believed present, filter 0.2X, 1X and 5X the basic filtration volumes. If the pollution levels are believed due primarily to human sources, use 1X, 5X and 25X the basic filtration volumes.

b If prior streptococcus data are available, use the equation

$$\text{Basic filtration volume in ml} = 100 \times \frac{60}{\text{Av. Streptococcus count per 100 ml}}$$

and filter 0.2X, 1X, and 5X the basic filtration volume for streptococci.

REFERENCES

1 The Federal Register. October 23, 1956, pp 8110-11; and March 1, 1957. p 1271.

2 Clark, H.F., Kabler, P.W., and Geldreich, E.E. Advantages and Limitations of the Membrane Filter Procedure. Water and Sewage Works. September 1957.

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Descriptors: Bacteria, Sample Testing Procedures, Filter, Membrane

DETAILED MEMBRANE FILTER METHODS

I BASIC PROCEDURES

A Introduction

Successful application of membrane filter methods requires development of good routine operational practices. The detailed basic procedures described in this Section are applicable to all membrane filter methods in water bacteriology for filtration, incubation, colony counting, and reporting of results. In addition, equipment and supplies used in membrane filter procedures described here are not repeated elsewhere in this text in such detail.

Workers using membrane filter methods for the first time are urged to become thoroughly familiar with these basic procedures and precautions.

B General Supplies and Equipment List

Table 1 is a check list of materials.

C "Sterilizing" Media

Set tubes of freshly prepared medium in a boiling waterbath for 10 minutes. This method suffices for medium in tubes up to 25 × 150 mm. Frequent agitation is needed with media containing agar.

Alternately, coliform media can be directly heated on a hotplate to the first bubble of boiling. Stir the medium frequently if direct heat is used, to avoid charring the medium.

Do not sterilize in the autoclave.

D General Laboratory Procedures with Membrane Filters

1 Prepare data sheet

Minimum data required are: sample identification, test performed including media and methods, sample filtration volumes, and the bench numbers assigned to individual membrane filters.

2 Disinfect the laboratory bench surface.

Use a suitable disinfectant solution and allow the surface to dry before proceeding.

3 Set out sterile culture containers in an orderly arrangement.

4 Label the culture containers.

Numbers correspond with the filter numbers shown on the data sheet.

5 Place one sterile absorbent pad* in each culture container, unless an agar medium is being used.

Use sterile forceps for all manipulations of absorbent pads and membrane filters. Forceps sterility is maintained by storing the working tips in about 1 inch of methanol or ethanol. Because the alcohol deteriorates the filter, dissipate it by burning before using the forceps. Avoid heating the forceps in the burner as hot metal chars the filter.

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*When an agar medium is used, absorbent pads are not used. The amount of medium should be sufficient to make a layer approximately 1/8" deep in the culture container. In the 50 mm plastic culture containers this corresponds to approximately 6-8 ml of culture medium.

NOTE: Mention of commercial products and manufacturers does not imply endorsement by the Office Of Water Programs, Environmental Protection Agency.

Table 1. EQUIPMENT, SUPPLIES AND MEDIA

Item	Total Coliforms			Fecal Coliform	Fecal Streptococcus	Verified Tests
	M-Endo Broth	L. E. S. Coliform	Delayed Coliform			
Funnel unit assemblies	X	X	X	X	X	
Ring stand, with about a 3" split ring, to support the filtration funnel	X	X	X	X	X	
Forceps, smooth tips, type for MF work	X	X	X	X	X	
Methanol, in small wide-mouthed bottles, about 20 ml for sterilizing forceps	X	X	X	X	X	
Suction flasks, glass, 1 liter, mouth to fit No. 8 stopper	X	X	X	X	X	
Rubber tubing, 2-3 feet, to connect suction flask to vacuum services, latex rubber 3/16" I.D. by 3/32" wall	X	X	X	X	X	
Pinch clamps strong enough for tight compression of rubber tubing above	X	X	X	X	X	
Pipettes, 10 ml, graduated, Mohr type, sterile, dispense 10 per can per working space per day. (Resterilize daily to meet need).	X	X	X	X	X	
Pipettes, 1 ml, graduated, Mohr type, sterile, dispense 24 per can per working space per day. (Resterilize daily to meet need).	X	X	X	X	X	
Pipette boxes, sterile, for 1 ml and 10 ml pipettes (sterilize above pipettes in these boxes).	X	X	X	X	X	
Cylinders, 100 ml graduated, sterile, (resterilize daily to meet need).	X	X	X	X	X	
Jars, to receive used pipettes	X	X	X	X	X	
Gas burner, Bunsen or similar laboratory type	X	X	X	X	X	X
Wax pencils, red, suitable for writing on glass	X	X	X	X	X	
Sponge in dilute iodine, to disinfect the desk tops	X	X	X	X	X	
Membrane filters (white, grid marked, sterile, and suitable pore size for microbiological analysis of water)	X	X	X	X	X	
Absorbent pads for nutrient, (47 mm in diameter), sterile, in units of 10 pads per package. Not required if medium contains agar.	X	X	X	X	X	
Petri dishes, disposable, plastic, 50 × 12 mm, sterile	X	X	X	X	X	
Waterbath incubator 44.5 ± 0.2°C				X		
Vegetable crispers, or cake boxes, plastic, with tight fitting covers, for membrane filter incubations	X	X	X		X	
Fluorescent lamp, with extension cord.	X	X	X	X	X	X
Ring stand, with clamps, utility type	X	X	X	X	X	

Table 1. EQUIPMENT, SUPPLIES AND MEDIA (Cont'd)

	Total Coliforms					
Item	M-Endo Broth	L. E. S. Coliform	Delayed Coliform	Fecal Coliform	Fecal Streptococcus	Verified Tests
Half-round glass paper weights for colony counting,with lower half of a 2-oz metal ointment box	X	X	X	X	X	
Hand tally, single unit acceptable, hand or desk type	X	X	X	X	X	
Stereoscopic (dissection) microscope, magnification of 10X or 15X, preferable binocular wide field type	X	X	X	X	X	X
Bacteriological inoculating needle						X
Wire racks for culture tubes, 10 openings by five openings preferred, dimensions overall approximately 6" × 12"						X
Phenol Red Lactose Broth in 16 × 150 mm fermentation tubes with metal caps, 10 ml per tube						X
Eosin Methylene Blue Agar (Levine) in petri plates, prepared ready for use						X
Nutrient agar slants, in screw capped tubes, 16 × 126 mm						X
Gram stain solutions, 4 solutions per complete set						X
Microscope, compound, binocular, with oil immersion lens, microscope lamp and immersion oil						X
Microscope slides, new, clean, 1" × 3" size						X
Water proof plastic bags for fecal coliform culture dish incubation				X		
M-Endo medium, MF dehydrated medium in 25 × 95 mm flat bottomed screw-capped glass vials, 1.44 g per tube, sufficient for 30 ml of medium	X		X			
Ethanol, 95% in small bottles or screw-capped tubes, about 20 ml per tube	X	X	X			
Sodium benzoate solution, 12% aqueous, in 25 × 150 mm screw-capped tubes, about 10 ml per tube			X			
L. E. S. Endo Agar MF, dehydrated M-Endo medium, 0.36 g per 25 × 95 mm flat bottomed screw-capped glass vial, plus 0.45 g agar, for 30 ml		X				
Lactose Lauryl Sulfate Tryptose Broth in 25 × 150 mm test tube without included gas tube, about 25 ml, for enrichment in L. E. S. method		X				

Table 1. EQUIPMENT, SUPPLIES AND MEDIA (Cont'd)

Item	Total Coliforms					
	M-Endo Broth	L. E. S. Coliform	Delayed Coliform	Fecal Coliform	Fecal Streptococcus	Verified Test
M-FC Broth for fecal coliform, dehydrated medium in 25 × 95 mm flat bottomed screw-capped glass vials, 1.11 g per tube, sufficient for 30 ml of culture medium				X		
Rosolic acid, 1% solution, in 0.2N NaOH, in 25 × 150 mm flat bottomed screw-capped tubes, about 5 ml per tube, freshly prepared				X		
KF Agar, dehydrated medium in 25 × 150 mm screw-capped tubes, sufficient for 30 ml, 2.3g per tube					X	
Dilution bottles, 6-oz, preferable boro-silicate glass, with screw-cap (or rubber stopper protected by paper), each containing 99 ml of sterile phosphate buffered distilled water	X	X	X	X	X	
Electric hot plate surface	X	X	X	X	X	
Beakers, 400 - 600 ml (for water-bath in preparation of membrane filter culture media)	X	X	X	X	X	
Crucible tongs, to be used at electric hot plates, for removal of hot tubes of culture media for boiling waterbath	X	X	X	X	X	

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6 Deliver enough culture medium to saturate each absorbent pad, using a sterile pipette.

Exact quantities cannot be stated because pads and culture containers vary. Sufficient medium should be applied so that when the culture container is tipped, a good-sized drop of culture medium freely drains out of the absorbent pad.

7 Organize supplies and equipment for convenient sample filtration. In training courses, laboratory instructors will suggest useful arrangements; eventually the individual will select a system of bench-top organization most suited to his own needs. The important point in any arrangement is to have all needed equipment and supplies conveniently at hand, in such a pattern as to minimize lost time in useless motions.

8 Lay a sterile membrane filter on the filter holder, grid-side up, centered over the porous part of the filter support plate.

Membrane filters are extremely delicate and easily damaged. For manipulation, the sterile forceps should always grasp the outer part of the filter disk, outside the part of the filter through which the sample passes.

9 Attach the funnel element to the base of the filtration unit.

To avoid damage to the membrane filter, locking forces should only be applied at the locking arrangement. The funnel element never should be turned or twisted while being seated and locked to the lower element of the filter holding unit. Filter holding units featuring a bayonet joint and locking ring to join the upper element to the lower element require special care on the part of the operator. The locking ring should be turned sufficiently to give a snug fit, but should not be tightened excessively.

10 Shake the sample thoroughly.

11 Measure sample into the funnel with vacuum turned off.

The primary objectives here are: 1) accurate measurement of sample; and 2) optimum distribution of colonies on the filter after incubation. To meet these objectives, methods of measurement and dispensation to the filtration assembly are varied with different sample filtration volumes.

a With samples greater than 20 ml, measure the sample with a sterile graduated cylinder and pour it into the funnel. It is important to rinse this graduate with sterile buffered distilled water to preclude the loss of excessive sample volume. This should be poured into the funnel.

b With samples of 10 ml to 20 ml, measure the sample with a sterile 10 ml or 20 ml pipette, and pipette on a dry membrane in the filtration assembly.

c With samples of 2 ml to 10 ml, pour about 20 ml of sterile dilution water into the filtration assembly, then measure the sample into the sterile buffered dilution water with a 10 ml sterile pipette.

d With samples of 0.5 to 2 ml, pour about 20 ml of sterile dilution water into the funnel assembly, then measure the sample into the sterile dilution water in the funnel with a 1 ml or a 2 ml pipette.

e If a sample of less than 0.5 ml is to be filtered, prepare appropriate dilutions in sterile dilution water, and proceed as applicable in item c or d above.

When dilutions of samples are needed, always make the filtrations as soon as possible after dilution of the sample; this never should exceed

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30 minutes. Always shake sample dilutions thoroughly before delivering measured volumes.

12 Turn on the vacuum.

Open the appropriate spring clamp or valve, and filter the sample.

After sample filtration a few droplets of sample usually remain adhered to the funnel walls. Unless these droplets are removed, the bacteria contained in them will be a source of contamination of later samples. (In laboratory practice the funnel unit is not routinely sterilized between successive filtrations of a series). The purpose of the funnel rinse is to flush all droplets of a sample from the funnel walls to the membrane filter. Extensive tests have shown that with proper rinsing technique, bacterial retention on the funnel walls is negligible.

13 Rinse the sample through the filter.

After all the sample has passed through the membrane filter, rinse down the sides of the funnel walls with at least 20 ml of sterile dilution water. Repeat the rinse twice after all the first rinse has passed through the filter. Cut off suction on the filtration assembly.

14 Remove the funnel element of the filter holding unit.

If a ring stand with split ring is used, hang the funnel element on the ring; otherwise, place the inverted funnel element on the inner surface of the wrapping material. This requires care in opening the sterilized package, but it is effective as a protection of the funnel ring from contamination.

15 Take the membrane filter from the filter holder and carefully place it, grid-side up on the medium.

Check that no air bubbles have been trapped between the membrane filter and the underlying absorbent pad or agar. Relay the membrane if necessary.

16 Place in incubator after finishing filtration series.

Invert the containers. The immediate atmosphere of the incubating membrane filter must be at or very near 100% relative humidity.

17 Count colonies which have appeared after incubating for the prescribed time.

A stereoscopic microscope magnifying 10-15 times and careful illumination give best counts.

For reporting results, the computation is:

$$\text{bacteria}/100\text{ ml} = \frac{\text{No. colonies counted} \times 100}{\text{Sample volume filtered in ml}}$$

Example:

A total of 36 colonies grew after filtering a 10 ml sample. The number reported is:

$$\frac{36\text{ colonies}}{10\text{ ml}} \times 100 = 360\text{ per }100\text{ ml}$$

Report results to two significant figures.

Example:

A total of 40 colonies grew after filtering a 3 ml sample.

This calculation gives:

$$\frac{40\text{ colonies}}{3\text{ ml}} \times 100 = 1333.33\text{ per }100\text{ ml}$$

But the number reported should be 1300 per 100 ml.

II MF LABORATORY TESTS FOR COLIFORM GROUP

A Standard Coliform Test (Based on M-Endo Broth MF)

1 Culture medium

a M-Endo Broth MF Difco 0749-02 or the equivalent BBL M-Coliform Broth 01-494

Preparation of Culture Medium (M-Endo Broth) for Standard MF Coliform Test

Yeast extract	1.5	g
Casitone or equivalent	5.0	g
Thiopeptone or equivalent	5.0	g
Tryptose	10.0	g
Lactose	12.5	g
Sodium desoxycholate	0.1	g
Dipotassium phosphate	4.375	g
Monopotassium phosphate	1.375	g
Sodium chloride	5.0	g
Sodium lauryl sulfate	0.05	g
Basic fuchsin (bacteriological)	1.05	g
Sodium sulfite	2.1	g
Distilled water (containing 20.0 ml ethanol)	1000 ml	

This medium is available in dehydrated form and it is recommended that the commercially available medium be used in preference to compounding the medium of its individual constituents.

To prepare the medium for use, suspend the dehydrated medium at the rate of 48 grams per liter of water containing ethyl alcohol at the rate of 20 ml per liter.

As a time-saving convenience, it is recommended that the laboratory worker preweigh the dehydrated medium in closed tubes for several days, or even weeks, at one operation. With this system, a large number of increments of dehydrated medium (e.g., 1.44 grams), sufficient for some convenient (e.g., 30 ml) volume of finished culture medium are weighed and dispensed into screw-capped culture tubes, and stored until needed. Storage should preferably be in a darkened disiccator.

A supply of distilled water containing 20 ml stock ethanol per liter can be maintained.

When the medium is to be used, it is reconstituted by adding 30 ml of the distilled water-ethanol mixture per tube of pre-weighed dehydrated culture medium.

b Medium is "sterilized" as directed in I, C.

c Finished medium can be retained up to 96 hours if kept in a cool, dark place. Many workers prefer to reconstitute fresh medium daily.

2 Filtration and incubation procedures are as given in I, D.

Special instructions:

a For counting, use the wide field binocular dissecting microscope, or simple lens. For illumination, use a light source perpendicular to the plane of the membrane filter. A small fluorescent lamp is ideal for the purpose.

b Coliform colonies have a "metallic" surface sheen under reflected light which may cover the entire colony, or it may appear only in the center. Non-coliform colonies range from colorless to pink, but do not have the characteristic sheen.

c Record the colony counts on the data sheet, and compute the coliform count per 100 ml of sample.

B Standard Coliform Tests (Based on L. E. S. Endo Agar)

The distinction of the L. E. S. count is a two hour enrichment incubation on LST broth. M-Endo L. E. S. medium is used as agar rather than the broth.

1 Preparation of culture medium (L. E. S. Endo Agar) for L. E. S. coliform test

a Formula from McCarthy, Delaney, and Grasso (2)

Bacto-Yeast Extract	1.2	g
Bacto-Casitone	3.7	g
Bacto-Thiopeptone	3.7	g
Bacto-Tryptose	7.5	g
Bacto-Lactose	9.4	g
Dipotassium phosphate	3.3	g
Monopotassium phosphate	1.0	g
Sodium chloride	3.7	g
Sodium desoxycholate	0.1	g
Sodium lauryl sulfate	0.05	g
Sodium sulfite	1.6	g
Bacto-Basic fuchsin	0.8	g
Agar	15	g
Distilled water (containing 20 ml ethyl alcohol)	1000	ml

b To rehydrate the medium, suspend 51 grams in the water-ethyl alcohol solution.

c Medium is "sterilized" as directed in I, C.

d Pour 4-6 ml of freshly prepared Agar into the smaller half of the container. Allow the medium to cool and solidify.

2 Procedures for filtration and incubation

a Lay out the culture dishes in a row or series of rows as usual. Place these with the upper (lid) or top side down.

b Place one sterile absorbent pad in the larger half of each container (lid). Use sterile forceps for all manipulations of the pads. (Agar occupies smaller half or bottom).

c Using a sterile pipette, deliver enough single strength lauryl sulfate tryptose broth to saturate the pad only. Avoid excess medium.

d Follow general procedures for filtering in I, D. Place filters on pad with lauryl sulfate tryptose broth.

e Upon completion of the filtrations, invert the culture containers and incubate at 35° C for 1 1/2 to 2 hours.

3 2-hour procedures

a Transfer the membrane filter from the enrichment pad in the upper half to the agar medium in the lower half of the container. Carefully roll the membrane onto the agar surface to avoid trapping air bubbles beneath the membrane.

b Removal of the used absorbent pad is optional.

c The container is inverted and incubated 22 hours ± 2 hours ± 0.5°C.

4 Counting procedures are as in I, D.

5 L. E. S. Endo Agar may be used as a single-stage medium (no enrichment step) in the same manner as M-Endo Broth, MF.

C Delayed Incubation Coliform Test

This technique is applicable in situations where there is an excessive delay between sample collection and plating. The procedure is unnecessary when the interval between sample collection and plating is within acceptable limits.

1 Preparation of culture media for delayed incubation coliform test

a Preservative media M-Endo Broth base

To 30 ml of M-Endo Broth MF prepared in accordance with directions in II, A, 1 of this outline, add 1.0 ml of a sterile 12% aqueous solution of sodium benzoate.

L. E. S. MF Holding Medium-Coliform: Dissolve 12.7 grams in 1 liter of distilled water. No heating is necessary. Final pH 7.1 ± 0.1. This medium contains sodium benzoate.

b Growth media

M-Endo Broth MF is used, prepared as described in II, A, 1 earlier in this outline. Alternately, L. E. S. Endo Medium may be used.

2 General filtration followed is in I, D.

Special procedures are:

a Transfer the membrane filter from the filtration apparatus to a pad saturated with benzoated M-Endo Broth.

b Close the culture dishes and hold in a container at ambient temperature. This may be mailed or transported to a central laboratory. The mailing or transporting tube should contain accurate transmittal data sheets which correspond to properly labeled dishes.

Transportation time, in the case of mailed containers, should not exceed three days to the time of reception by the testing laboratory.

c On receipt in the central laboratory, unpack mailing carton, and lay out the culture containers on the laboratory bench.

d Remove the tops from the culture containers. Using sterile forceps, remove each membrane and its absorbent pad to the other half of the culture container.

e With a sterile pipette or sterile absorbent pad, remove preservative medium from the culture container.

f Place a sterile absorbent pad in each culture container, and deliver enough freshly prepared M-Endo Broth to saturate each pad.

g Using sterile forceps, transfer the membrane to the new absorbent pad containing M-Endo Broth. Place the membrane carefully to avoid entrapment of air between the membrane and the underlying absorbent pad. Discard the absorbent pad containing preservative medium.

h After incubation of 20 ± 2 hours at 35°C, count colonies as in the above section A, 2.

i If L. E. S. Endo Agar is used, the steps beginning with (e) above are omitted; and the membrane filter is removed from the preservative medium and transferred to a fresh culture container with L. E. S. Endo Agar, incubated, and colonies counted in the usual way.

D Verified Membrane Filter Coliform Test

This procedure applies to identification of colonies growing on Endo-type media used for determination of total coliform counts. Isolates from these colonies are studied for gas production from lactose and typical coliform morphology. In effect, the procedure corresponds with the Completed Test stage of the multiple fermentation tube test for coliforms.

Procedure:

1 Select a membrane filter bearing several well-isolated coliform-type colonies.

2 Using sterile technique, pick all colonies in a selected area with the inoculation needle, making transfers into tubes of phenol red lactose broth (or lauryl sulfate tryptose lactose

broth). Using an appropriate data sheet record the interpretation of each colony, using, for instance, "C" for colonies having the typical color and sheen of coliforms; "NC" for colonies not conforming to coliform colony appearance on Endotype media.

3 Incubate the broth tubes at 35° C ± 0.5°C.

4 At 24 hours:

a Read and record the results from the lactose broth fermentation tubes. The following code is suggested:

Code	
O	No indication of acid or gas production, either with or without evidence of growth.
A	Evidence of acid but not gas (applies only when a pH indicator is included in the broth medium)
G	Growth with production of gas. If pH indicator is used, use symbol AG to show evidence of acid. Gas in any quantity is a positive test.

b Tubes not showing gas production are returned to the 35° C incubator.

c Gas-positive tubes are transferred as follows:

1) Prepare a streak inoculation on EMB agar for colony isolation, and using the same culture.

2) Inoculate a nutrient agar slant.

3) Incubate the EMB agar plates and slants at 35° C ± 0.5°C

5 At 48 hours:

a Read and record results of lactose broth tubes which were negative at 24 hours and were returned for further incubation.

b Gas-positive cultures are subjected to further transfers as in 4c. Gas-negative cultures are discarded without further study; they are coliform- negative.

c Examine the cultures transferred to EMB agar plates and to nutrient agar slants, as follows:

1) Examine the EMB agar plate for evidence of purity of culture; if the culture represents more than one colony type, discard the nutrient agar culture and reisolate each of the representative colonial types on the EMB plate and resume as with 4c for each isolation. If purity of culture appears evident, continue with c (2) below.

2) Prepare a smear and Gram stain from each nutrient agar slant culture. The Gram stain should be made on a culture not more than 24 hours old. Examine under oil immersion for typical coliform morphology, and record results.

6 At 72 hours:

Perform procedures described in 5c above, and record results.

7 Coliform colonies are considered verified if the procedures demonstrate a pure culture of bacteria which are gram negative nonspore-forming rods and produce gas from lactose at 35° C within 48 hours.

E Fecal Coliform Count (Based on M-FC Broth Base)

The count depends upon growth on a special medium at 44.5 ± 0.2°C.

1 Preparation of Culture Medium (M-FC Broth Base) for Fecal Coliform Count

a Composition

Tryptose	10.0 g
Proteose Peptone No. 3	5.0 g
Yeast extract	3.0 g
Sodium chloride	5.0 g
Lactose	12.5 g
Bile salts No. 3	1.5 g
Rosolic acid* (Allied Chemical)	10.0 ml
Aniline blue (Allied Chemical)	0.1 g
Distilled water	1000 ml

b To prepare the medium dissolve 37.1 grams in a liter of distilled water which contains 10 ml of 1% rosolic acid (prepared in 0.2 N NaOH).

Fresh solutions of rosolic acid give best results. Discard solutions which have changed from dark red to orange.

c To sterilize, heat to boiling as directed in I, C.

d Prepared medium may be retained up to 4 days in the dark at 2-8° C.

2 Special supplies

Small water proof plastic sacks capable of being sealed against water with capacity of 3 to 6 culture containers.

3 Filtration procedures are as given in I, D.

4 Elevated temperature incubation

a Place fecal coliform count membranes at 44.5 ± 0.2°C as rapidly as possible.

Filter membranes for fecal coliform counts consecutively and immediately place them in their culture containers. Insert as many as six culture containers all oriented in the same way (i.e., all grid sides facing the same direction) into the sacks and seal. Tear off the perforated top, grasp the side wires, and twirl the sack to roll the open end inside the folds of sack. Then submerge the sacks with culture containers inverted beneath the surface of a 44.5 ± 0.2°C waterbath.

b Incubate for 22 ± 2 hours.

5 Counting procedures

Examine and count colonies as follows:

a Use a wide field binocular dissecting microscope with 5 - 10X magnification.

b Low angle lighting from the side is advantageous.

c Fecal coliform colonies are blue, generally 1-3 mm in diameter.

d Record the colony counts on the data sheet, and report the fecal coliform count per 100 ml of sample. (I, D, 17 illustrates method)

III TESTS FOR FECAL STREPTOCOCCAL GROUP-MEMBRANE FILTER METHOD

A 48 hour incubation period on a choice of two different media, giving high selectivity for fecal streptococci, are the distinctive features of the tests.

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*Prepare 1% solution of rosolic acid in 0.2 N NaOH. This dye is practically insoluble in water.

A Test for Members of Fecal Streptococcal Group based on KF-Agar

1 Preparation of the culture medium

a Formula: (The dehydrated formula of Bacto 0496 is shown, but equivalent constituents from other sources are acceptable). Formula is in grams per liter of reconstituted medium.

Bacto proteose peptone #3	10.0	g
Bacto yeast extract	10.0	g
Sodium chloride (reagent grade)	5.0	g
Sodium glycerphosphate	10.0	g
Maltose (CP)	20.0	g
Lactose (CP)	1.0	g
Sodium azide (Eastman)	0.4	g
Sodium carbonate (Na_2CO_3 reagent grade)	0.636	g
Brom cresol purple (water soluble)	0.015	g
Bacto agar	20.0	g

b Reagent

2, 3, 5-Triphenyl tetrazolium chloride reagent (TPTC)

This reagent is prepared by making a 1% aqueous solution of the above chemical passing it through a Seitz filter or membrane filter. It can be kept in the refrigerator in a screw-capped tube until used.

c The dehydrated medium described above is prepared for laboratory use as follows:

Suspend 7.64 grams of the dehydrated medium in 100 ml of distilled water in a flask with an aluminum foil cover.

Place the flask in a boiling waterbath, melt the dehydrated medium, and leave in the boiling waterbath an addional 5 minutes.

Cool the medium to 50°-60° C, add 1.0 ml of the TPTC reagent, and mix.

For membrane filter studies, pour 5-8 ml in each 50 mm glass or plastic culture dish or enough to make a layer approximately 1/8" thick. Be sure to pour plates before agar cools and solidifies.

For plate counts, pour as for standard agar plate counts.

NOTE: Plastic dishes containing media may be stored in a dark, cool place up to 30 days without change in productivity of the medium, provided that no dehydration occurs. Plastic dishes may be incubated in an ordinary air incubator. Glass dishes must be incubated in an atmosphere with saturated humidity.

2 Apparatus, and materials as given in Table 1.

3 General procedure is as given in I.

Special instructions

a Incubate 48 hours, inverted with 100% relative humidity after filtration.

b After incubation, remove the cultures from the incubator, and count colonies under wide field binocular dissecting microscope, with magnification set at 10X or 20X. Fecal streptococcus colonies are pale pink to dark wine-color. In size they range from barely visible to approximately 2mm in diameter. Colorless colonies are not counted.

c Report fecal streptococcus count per 100 ml of sample. This is computed as follows:

No. fecal streptococci per 100 ml =

$$\frac{\text{No. fecal streptococcus colonies}}{\text{Sample filtration volume in ml}} \times 100$$

B Verification of Streptococcus Colonies

1 Verification of colony identification may be required in waters containing large numbers of Micrococcus organisms. This has been noted particularly with bathing waters, but the problem is by no means limited to such waters.

2 A verification procedure is described in "Standard Methods for the Examination of Water and Wastewater," 14th ed. (1975). The worker should use this reference for the step-by-step procedure.

IV PROCEDURES FOR USE OF MEMBRANE FILTER FIELD UNITS

A Culture Media

1 The standard coliform media used with laboratory tests are used.

2 To simplify field operations, it is suggested that the medium be sent to the field, preweighed, in vials or capped culture tubes. The medium then requires only the addition of a suitable volume of distilled water-ethanol prior to sterilization.

3 Sterilization procedures in the field are the same as for laboratory methods.

4 Laboratory preparation of the media, ready for use, would be permissible provided that the required limitations on time and conditions of storage are met.

B Operation of Millipore Water Testing Kit, Bacteriological

1 Supporting supplies and equipment are the same as for the laboratory procedures.

2 Set the incubator voltage selector switch to the voltage of the available supply, turn on the unit and adjust as necessary to establish operating incubator temperature at 35 ± 0.5° C.

3 Sterilize the funnel unit assembly by exposure to formaldehyde or by immersion in boiling water. If a laboratory autoclave is available, this is preferred.

Formaldehyde is produced by soaking an asbestos ring (in the funnel base) with methanol, igniting, and after a few seconds of burning, closing the unit by placing the stainless steel flask over the funnel and base. This results in incomplete combustion of the methanol, whereby formaldehyde is produced. Leave the unit closed for 15 minutes to allow adequate exposure to formaldehyde.

4 Filtration and incubation procedures correspond with laboratory methods.

5 The unit is supplied with a booklet containing detailed step-by-step operational procedures. The worker using the equipment should become completely versed in its contents and application.

C Other commercially available field kits should be used according to manufacturer's instructions. It is emphasized that the required standards of performance are manditory for field devices as for laboratory equipment.

D Counting of Colonies on Membrane Filters

1 Equipment and materials

Membrane filter cultures to be examined

Illumination source

Simple lens, 2X to 6X magnification

Hand tally (optional)

2 Procedure

a Remove the cultures from the incubator and arrange them in numerical sequence.

b Set up illumination source as that light will originate from an area perpendicular to the plane of membrane filters being examined. A small fluorescent lamp is ideal for the purpose. It is highly desirable that a simple lens be attached to the light source.

c Examine results. Count all coliform and noncoliform colonies. Coliform and noncoliform colonies. Coliform colonies have a "metallic" surface sheen under reflected light, which may cover the entire colony or may appear only on the center. Noncoliform colonies range from colorless to pink or red, but do not have the characteristic "metallic" sheen.

d Enter the colony counts in the data sheets.

e Enter the coliform count per 100 ml of sample for each membrane having a countable number of coliform colonies. Computation is as follows:

No. coliform per 100 ml =

$$\frac{\text{No. coliform colonies on MF}}{\text{No. milliliters sample filtered}} \times 100$$

REFERENCES

1 Standard Methods for the Examination of Water and Wastewater. APHA, AWWA, WPCF 14th Edition. 1975.

2 McCarthy, J.A., Delaney, J.E. and Grasso, R.J. Measuring Coliforms in Water. Water and Sewage Works. 1961: R-426-31. 1961.

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COLONY COUNTING ON MEMBRANE FILTERS

I INTRODUCTION

On removal of membrane filter cultures from the incubator, the worker has several tasks to perform, leading to the reporting of results of the bacteriological examination. These steps, together with the selection and use of associated equipment, are considered in this discussion. The following topics are included:

A Precautions on removal of membrane filter cultures from the incubator.

B Selection of the best membrane filter for colony counting (when more than one membrane filter per sample was prepared, representing a graded series of sample increments.)

C Use of grid systems on filter surfaces as counting aids.

D Recognition and counting of desired colonies, including selection and use of optical equipment.

E Calculations for reporting number of test organisms per 100 ml of sample.

II REMOVAL OF CULTURE FROM INCUBATOR

A Incubation time and temperature recommendations should be closely adhered to. This applies particularly to total coliform counts. Some of our earlier training manuals have suggested counting of colonies after as few as 16 hours of incubation at 35°C. Currently, 22 ± 2 hours is preferred.

B All membrane filter cultures should be incubated in the inverted position, with measures to avoid loss of culture medium through leakage or evaporation. Sometimes an excessive amount of culture medium is applied initially, or additional moisture finds its way into the culture container during incubation. In such cases, when the culture is removed from the incubator, it should be turned "right side up" in such a way as to avoid flooding the filter with excess liquid. If excessive liquid is present, open the culture container cautiously, and pour off the excess.

C Drying Filters Before Colony Counts

1 Some workers advise opening all cultures (especially total coliform tests when Endo-type media are used) for a short time (15 minutes to 1 hour) for partial drying of coliform colonies before counting. Advocates of this step report that the typical surface sheen characteristic of coliform colonies is improved by this step.

2 Use of preliminary drying procedures is a matter of personal preference. In the opinion of the writer, the benefit of preliminary drying is at best debatable, and at worst, may interfere with subsequent study of the bacterial colonies. Correct use of acceptable lighting and optical equipment is a far more important factor in ease and accuracy of recognition of differentiated colonies.

III SELECTION OF ACCEPTABLE MEMBRANE FILTER CULTURE FOR EXAMINATION

A Non-Quantitative Tests

In bacteriologic examination of treated waters, where waters meeting requirements result in development of very few or no coliform colonies, the typical filtration volume is 100 ml, and but one filtration is made per sample. In this case, there is no problem: the one membrane

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filter preparation is the basis of bacteriologic evaluation of the sample.

B Quantitative Tests

1 When the bacteriological water quality standard is for some fixed limiting value, such as 70 per 100 ml for shellfish waters, again only a single sample filtration volume may be used. In such a case, the filtration of a single portion of 50 ml will show directly whether the water meets bacteriologic standards, or if the limiting standard is being exceeded.

2 On the other hand, if the objective of the test was to show how many coliforms were present per 100 ml of sample, then it is necessary to filter a series of sample increments from each sample, each increment being placed on a separate membrane filter. At the end of the incubation period, the series of membrane filters representing each sample must be inspected, with selection of the membrane filter bearing the number of colonies most suitable for reporting quantitative results. This is summarized in Table 1, below:

The lower limit of 20 is set arbitrarily, as a number below which statistically valid results become increasingly questionable with smaller numbers of colonies. The upper limits represent numbers above which interference from colony crowding, deposition of extraneous material, and other factors appear to result in increasingly questionable results. It is emphasized that these limiting values are empirical, based on laboratory observations alone, and do not represent results of theoretical calculations. It follows that it is quite possible, with some sample sources, to obtain acceptable quantitative results with colony counts higher than the recommendations, but the minimum limit of 20 colonies appears to apply to the majority of sample sources."

3 If no membrane filter bears a number of colonies within the recommended limits for the test, the worker has a choice between - a) collecting a new sample and repeating the test; and b) using whatever results actually were obtained, reporting an "educated guess" as to the number of organisms per 100 ml. In the latter case, it is most

Table 1. NUMBERS OF COLONIES ACCEPTABLE FOR QUANTITATIVE DETERMINATIONS

Test	Colony Counting Range		Remarks
	Minimum	Maximum	
Total coliform	20	80	200 limit overall
Fecal coliform	20	60	
Fecal streptococcus	20	100	

strongly urged that each result of this type be specifically identified with a qualifying statement, such as "Estimated count, based on non-ideal colony density on filter."

4 Sometimes two or more filters, of a series of filtration volumes from a sample, produce colony counts within the recommended counting range. Colony counts should be made on all such filters. See Section VI of this outline for calculations based on such results. These problems may arise from the selection of a too-close range of sample filtration volumes, from colony differentiation failures related to overcrowding on the filters, or from physico-chemical interference with colony development related to material in the sample deposited in or on the filters.

IV USE OF GRID SYSTEMS IN COLONY COUNTS

A Most manufacturers provide grid-imprinted membrane filters for bacteriologic use. The ink used in such filters must be biochemically inert to the test organisms, and, of course, must be applied in such a manner as not to degrade the quality of the filter. Examples of such gridding have appeared from various manufacturers as follows:

1 ... effective filtering area subdivided into squares equal to 1/100 the effective filtering area (when a filtering unit with funnel-diameter of 35 mm is used).

2 ... grid markings which subdivide the effective filtering area into squares equal to 1/100 the effective filtering area (9.6 cm^2 for 47 mm diameter filters).

3 ... filters subdivided so that each square of the grid represents 1/60 of the effective filtering area.

B Some special studies may require use of membrane filters without grid markings. For example, the ink in some filters prevents growth of Brucella melitensis. In such cases it may be necessary to improvise a viewing grid which can be placed over the culture after incubation and colony development.

C Applications of Grids

1 The grid dimensions are of no particular significance in colony counting, provided that their size permits easy and continuous orientation in counting of colonies. To be sure, a rough estimate of the total number of differentiated colonies on a filter is possible by counting a representative number of squares and multiplying colony count by the appropriate factor. For example, with many filters, colonies in ten squares can be counted, multiplied by 10, and the product is a rough estimate of the total number on the entire filter. It is emphasized that such procedure is for rough estimates only, and should not be condoned in quantitative work with membrane filters.

2 The primary usefulness of the grid system is for orientation during the counting procedure. Some colonies will touch lines on a grid system, and a uniform practice must be established to avoid missing some colonies or counting others twice. The procedure used by the writer is as follows:

a Counts are made in an orderly back-and-forth sweep, from top to bottom of the filter. See Figure 1.

b Inevitably, some colonies will be in contact with grid lines. A suggested routine procedure for counting colonies in contact with lines is indicated in Figure 2. Colonies are counted in the squares indicated by the arrows, and no effort is made to decide whether "most of the colony" is in one or the other square.

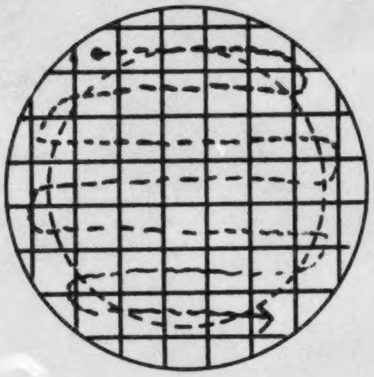

Figure 1. The dashed circle indicates the effective filtering area. The dashed back-and-forth line indicates the colony counting pathway.

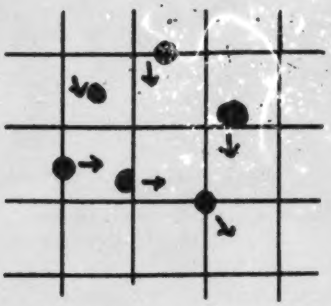

Figure 2. Enlarged portion of grid-marked square of filter, with various ways colonies can be in contact with grid-lines. Colonies are counted in squares indicated by the arrows.

V COUNTING OF COLONIES

A Equipment

1 A hand-tally is a useful device while counts are being made.

2 Optical assistance in colony counts is strongly recommended. Dependence on naked-eye counts often results in too-low results.

a Preferably, use a wide-field binocular dissecting microscope with magnification of 10X or 15X.

b Optionally, but less desirably, a simple lens with magnification at least 5X can be used, provided that acceptable illumination also is present.

3 Lighting equipment

a For coliform counting, a large light source is mandatory. Fluorescent lamps in housings permitting placement close to and as directly as possible over the membrane filter is the best lighting arrangement known to the writer. Incandescent lamps, whether simple light bulbs in a table lamp or in elaborate microscope lamp housings, are not satisfactory for coliform colony counting on membrane filters with Endo-type media.

b For fecal coliforms or fecal streptococci, the lighting requirements are not so severe; in this case almost any sufficiently bright light source, which can be placed above the filter (either at a high or at a low angle) will suffice.

4 Lighting arrangement and counting

a As above, for coliform counting, the fluorescent lamp should be at a high angle (as nearly as possible directly over the membrane filter) so placed that an image of the light source is reflected off the colony surfaces into the microscope lens system. Properly placed, the light will demonstrate the "golden metallic" surface luster of coliform colonies, which may cover the entire colony, or may appear only in an area in the center of the colony. The worker must learn to recognize the difference between the typical golden sheen of coliform colonies and the merely shiny surface of non-coliform colonies.

b Other types of colonies (fecal coliforms, fecal streptococci, etc.) do not require such rigid control of the light source. Low-angle lighting can be helpful, to give a relief of the colony profile from the colony surface. This is valuable with small colonies, such as frequently encountered in streptococcal studies. In such cases, almost any light source is acceptable, provided that it is bright enough and that it is applied from somewhere above the membrane filter.

c The typical appearance of various types of colonies is related to the culture media applied; therefore, this is not discussed in detail at this point. See the outlines on culture media and on laboratory procedures for specified indicator organisms for such information.

d In colony counting, count all colonies individually, even if they are in contact with each other (this is contrary to usual practice in colony counting in agar cultures in Petri dishes). Such colonies are recognized quite easily when a microscope is used for colony counting as recommended. Colonies which have grown into contact almost invariably show a very fine line of contact. The worker must learn to recognize the difference between two or more colonies which have grown into contact with each other, and single, irregularly shaped, colonies which sometimes develop on membrane filters. Such colonies almost invariably are associated with a fiber or particulate material deposited on the filter, and tend to develop along a path conforming to the shape and size of the fiber or particulates.

VI CALCULATIONS

A Counting Units

1 In reporting densities of indicator organisms (coliforms, fecal coliforms, fecal streptococci), bacterial counts always are reported in numbers per 100 ml. In standard practice, results are expressed to two significant figures. For example, if the calculation indicates 75,400, or even 75,444 organisms per 100 ml, the results would be reported as 75,000 per 100 ml in each case. (The digits 7 and 5 are the significant figures; the three zeros only locate the decimal point.)

2 When "total" bacterial counts are reported, common practice is to report in number per ml, not the number per 100 ml.

3 Quantitative work on enteric pathogens is, at this time, limited to reporting of occurrence of designated enteric pathogens, correlated with measured density of pollution indicating bacterial groups. At such time as the numerical determination of enteric pathogens becomes feasible, it is anticipated that reports will be in terms of count per 100 ml, or even larger volume units.

B Typical Calculations

1 Select the membrane filter bearing the acceptable number of colonies for reporting, and calculate indicators per 100 ml according to the general formula:

$$\text{No. indicator organisms per 100 ml} = \frac{\text{No. colonies of indicator organism}}{\text{No. ml of sample filtered}} \times 100$$

2 Example:

a Assume that for a total coliform count, volumes of 50, 15, 5, 1.5, and 0.5 ml produced coliform colony counts of 200, 110, 40, 10, and 5, respectively.

b First, the worker actually would not have counted coliform colonies on all these filters. He would have selected, by inspection, the membrane filter(s) most likely to have 20-80 coliform colonies, limiting actual counting to such colonies (this does take some practice and skill in making quick estimates, but comes with experience).

c Having selected the membrane filter probably most useful for reporting purposes, coliform colonies are counted according to accepted procedures, and the general formula is applied:

$$\text{Coliforms per 100 ml} = \frac{40}{5} \times 100$$

$$\text{Coliforms per 100 ml} = 800$$

C Special Situations in Calculating Densities of Indicator Organisms

1 Assume a coliform count in which the volumes of 1, 0.3, 0.1, 0.03, and 0.01 ml, respectively, produced coliform colony counts of TNTC, TNTC, 75, 30, and 8, respectively.

a Here, two sample volumes resulted in production of coliform colonies in the acceptable counting range.

b Suggestion: Compile the filtration volumes and colonies from both acceptable filters, as follows:

Volume, ml	Count
0.1	75
0.03	30
0.13	105

Calculate coliforms per 100 ml from the composite result:

$$\text{Coliforms per 100 ml} = \frac{105}{0.13} \times 100$$

$$\text{Coliforms per 100 ml} = 81{,}000$$

2 Assume a coliform count in which sample volumes of 1, 0.3, and .01 ml produced colony counts of 14, 3, and 0, respectively.

a Here, no colony count falls within recommended limits.

b Suggestion: Calculate on the basis of the most nearly acceptable value, and report with qualifying remark, thus:

Use 14 colonies from 1 ml of sample:

$$\frac{14}{1.0} \times 100 = 1400$$

Report: "Estimated Count, 1400 per 100 ml, based on non-ideal colony count".

3 Assume a coliform count in which the volumes 1, 0.3, and 0.01 ml produced coliform colony counts of 0, 0, and 0, respectively.

a Here, no actual calculation is possible, even for "estimate" reports.

b Suggestion: Calculate the number of estimated coliforms per 100 ml that would have been reported if there had been 1 coliform colony on the filter representing the largest filtration volume, thus:

Use 1 colony, and 1 ml: $\frac{1}{1} \times 100 = 100$

Report: "Less than 100 coliforms per 100 ml".

4 Assume a coliform count in which the volumes of 1, 0.3, and 0.01 ml produced coliform colony counts of TNC, 150, and 110 colonies.

a Here, all colony counts are above the recommended limits.

b Suggestion: Use Example 2, above, and report an estimated count based on non-ideal colony counts:

$$\frac{110}{0.01} \times 100 = 1{,}100{,}000$$

Report: "Coliform count estimated at 1,100,000 per 100 ml, based on non-ideal colony count".

5 Assume that, in Example 4, the volumes of 1.0, 0.3, and 0.01 ml, all produced too many coliform colonies to show separated colonies, and that the laboratory bench record showed TNTC (Too Numerous to Count).

Suggestion: Use 80 colonies as the basis of calculation with the smallest filtration volume, thus:

$$\frac{80}{0.01} \times 100 = 800,000$$

Report: "> 800,000 coliforms per 100 ml sample. Filters too crowded."

VII CONCLUSION

The foregoing discussion has presented a number of factors which determine the quantitative reliability of membrane filter results. It cannot be too strongly emphasized that the correct use of acceptable colony counting equipment is one of the most important single factors in successful application of membrane filter methods. Here, there is perhaps a greater exercise of personal skill and judgment than in any other aspect of membrane filter methodology. There is no substitute for practice and experience, supported by liberal use of supporting colony verification studies, to produce a skilled worker in colony counts on membrane filters.

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Descriptors: Filters, Membrane, Bacteria, Microorganisms, Measurement Testing Procedures

VERIFIED MEMBRANE FILTER TESTS

I INTRODUCTION

A The purpose of a verified membrane filter test procedure is to establish the validity of colony differentiation and interpretation in the test being applied. Specifically, a verified membrane filter test may prove useful 1) as a self-training device for new workers, 2) as a research tool in evaluation of new membrane filter media and procedures, or 3) to provide supporting evidence of colony interpretation in cases where the analytical results may be subject to professional or official challenge.

B Reduced to essentials, a verified membrane filter test consists of 1) interpretation of the colonies appearing on a selective, differential medium, 2) recovery of purified bacterial cultures from differentiated colonies, and 3) application of supplemental test procedures to determine the validity of the original interpretation of the membrane filter colonies.

C In this discussion, primary attention is given to a verified membrane filter coliform test. In addition, verification procedures are presented for members of the fecal coliform group and for fecal streptococci.

II VERIFIED TEST FOR MEMBERS OF THE COLIFORM GROUP

A An abbreviated procedure corresponds to the Confirmed Test of Standard Methods through use of lactose broth (or lactose lauryl tryptose broth) followed by confirmation in brilliant green lactose bile broth. The procedure is shown diagrammatically as follows:

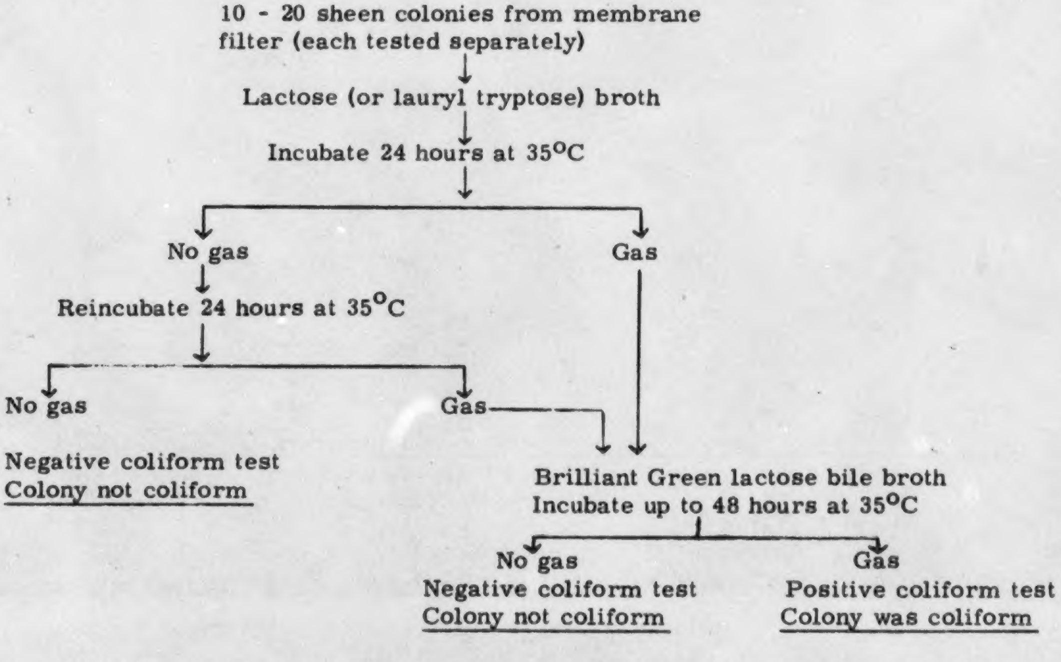
10 - 20 sheen colonies from membrane filter (each tested separately)
Lactose (or lauryl tryptose) broth
Incubate 24 hours at 35°C
No gas
Gas
Reincubate 24 hours at 35°C
No gas
Gas
Negative coliform test
Colony not coliform
Brilliant Green lactose bile broth
Incubate up to 48 hours at 35°C
No gas
Negative coliform test
Colony not coliform
Gas
Positive coliform test
Colony was coliform

Diagram 1. ABBREVIATED COLIFORM VERIFICATION PROCEDURE

B A more elaborate verification of membrane filter test for coliforms resembles the Completed Test of Standard Methods. The test is started in exactly the same way as the abbreviated test, and may be represented diagrammatically as a continuation from the lactose broth stage of Diagram 1. See Diagram 2.

C While the diagrams (1 and 2) are presented in terms of sheen colonies (interpreted as coliforms), the careful worker also should subject a similarly representative number of non-sheen colonies (judged to be noncoliforms) to the same test procedure. This will reveal whether the medium being studied fails to differentiate appreciable numbers of colonies which in reality are coliforms, even though they did not demonstrate the desired differential characteristic.

III VERIFICATION OF FECAL COLIFORM TESTS ON MEMBRANE FILTERS

A The procedure described here is based on the principle that, with use of m-FC Broth and incubation in a water bath at 44.5°C for 24 hours, fecal coliform colonies on membrane filters develop a blue color, (sometimes a greenish-blue). Extraneous bacteria are believed to fail to develop colonies, or else consist of such colonies develop some color other than the blue color of fecal coliforms (colorless, buff- or brownish-color, or even red colonies may develop on the medium).

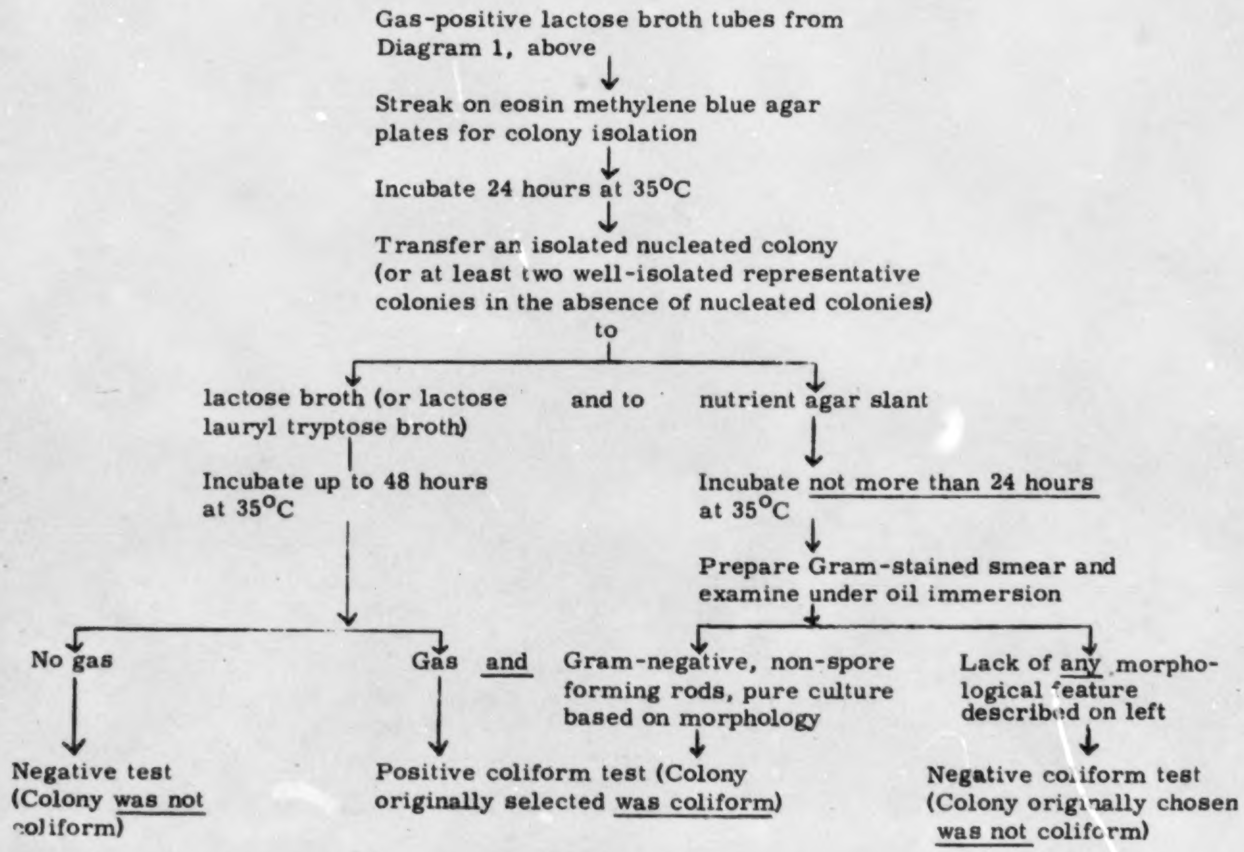
Gas-positive lactose broth tubes from Diagram 1, above
Streak on eosin methylene blue agar plates for colony isolation
Incubate 24 hours at 35°C
Transfer an isolated nucleated colony (or at least two well-isolated representative colonies in the absence of nucleated colonies)
to
lactose broth (or lactose lauryl tryptose broth)
and to
nutrient agar slant
Incubate up to 48 hours at 35°C
Incubate not more than 24 hours at 35°C
Prepare Gram-stained smear and examine under oil immersion
No gas
Gas
and
Gram-negative, non-spore forming rods, pure culture based on morphology
Lack of any morphological feature described on left
Negative test (Colony was not coliform)
Positive coliform test (Colony originally selected was coliform)
Negative coliform test (Colony originally chosen was not coliform)

Diagram 2. EXTENDED COLIFORM VERIFICATION PROCEDURE

B The verified test for fecal coliforms is indicated in Diagram 3, below:

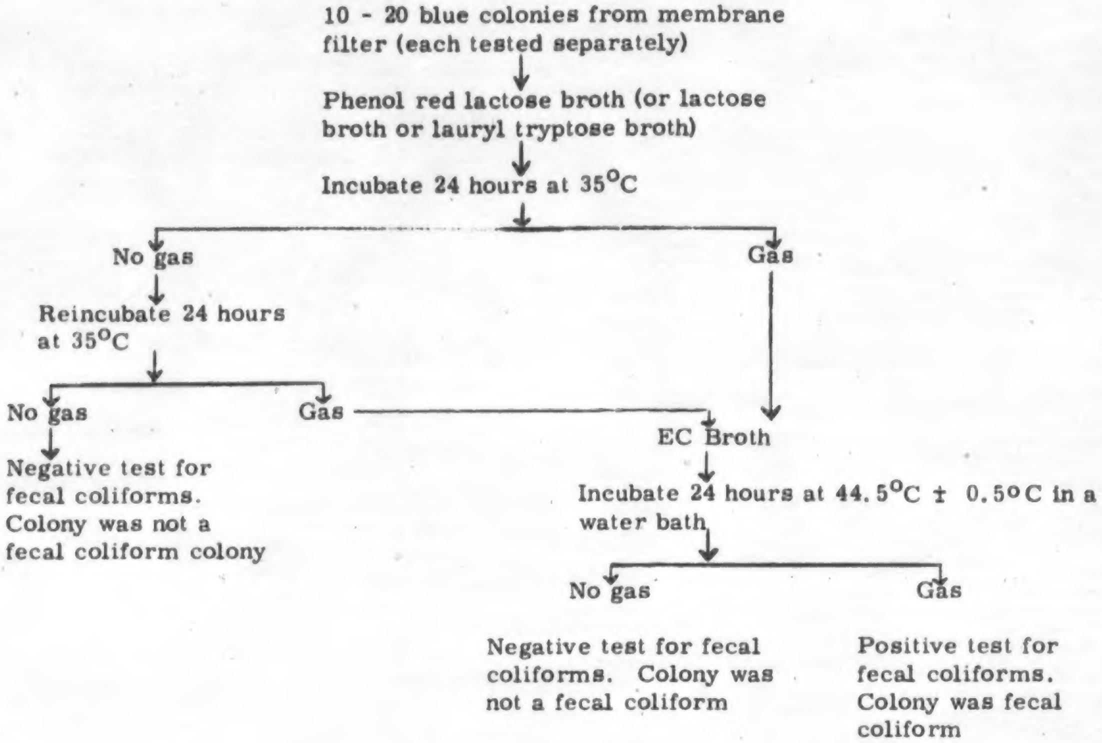
10 - 20 blue colonies from membrane filter (each tested separately)
Phenol red lactose broth (or lactose broth or lauryl tryptose broth)
Incubate 24 hours at 35°C
No gas
Gas
Reincubate 24 hours at 35°C
No gas
Gas
Negative test for fecal coliforms. Colony was not a fecal coliform colony
EC Broth
Incubate 24 hours at 44.5°C ± 0.5°C in a water bath
No gas
Gas
Negative test for fecal coliforms. Colony was not a fecal coliform
Positive test for fecal coliforms. Colony was fecal coliform

Diagram 3. A VERIFICATION PROCEDURE FOR FECAL COLIFORMS ON MEMBRANE FILTERS

IV VERIFICATION OF FECAL STREPTOCOCCUS COLONIES ON MEMBRANE FILTERS

A The procedure is used in the evaluation of results from a medium similar to the m-Enterococcus Agar (Slanetz) described in the current edition of Standard Methods. The membrane filter procedure utilizes 48 hour incubation at 35°C, and colonies which are pink to red, either in their entirety or only in their centers, are regarded as fecal streptococci. Most such colonies are 1-2 mm in diameter, and some may be larger. Occasionally, some samples may be encountered in which numerous extremely small colonies, approximately 0.1 mm in diameter, are present in great numbers. Almost invariably, these are not fecal streptococci. See diagram 4 for a representation of a verification test.

V CALCULATIONS BASED ON VERIFICATION STUDIES

A A percent verification can be determined for any colony-validation test:

$$\text{Percent verification} = \frac{\text{No. of colonies meeting verification test}}{\text{No. of colonies subjected to verification}} \times 100$$

10 - 20 Pink to red colonies from membrane filter (each tested separately)

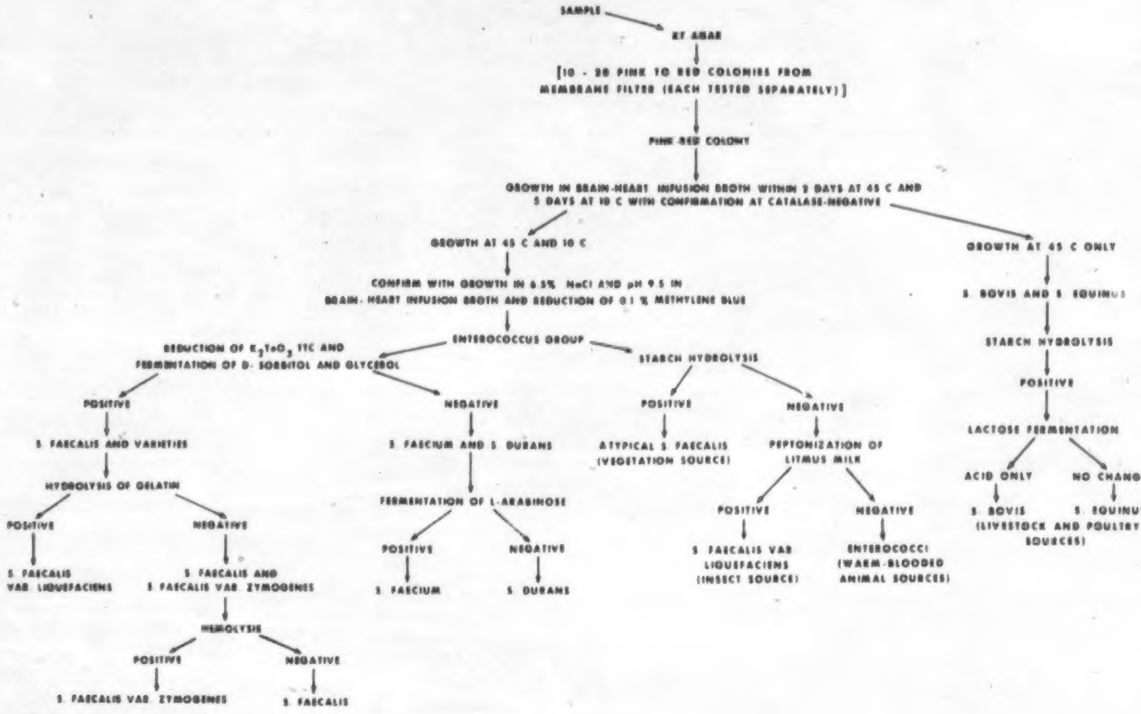
SAMPLE
KF AGAR
[10 - 20 PINK TO RED COLONIES FROM MEMBRANE FILTER (EACH TESTED SEPARATELY)]
PINK-RED COLONY
GROWTH IN BRAIN-HEART INFUSION BROTH WITHIN 2 DAYS AT 45 C AND 5 DAYS AT 10 C WITH CONFIRMATION AT CATALASE-NEGATIVE
GROWTH AT 45 C AND 10 C
CONFIRM WITH GROWTH IN 6.5% NaCl AND pH 9.5 IN BRAIN-HEART INFUSION BROTH AND REDUCTION OF 0.1 % METHYLENE BLUE
ENTEROCOCCUS GROUP
REDUCTION OF K2TeO3 TTC AND FERMENTATION OF D-SORBITOL AND GLYCEROL
POSITIVE
S. FAECALIS AND VARIETIES
HYDROLYSIS OF GELATIN
POSITIVE
S. FAECALIS VAR. LIQUEFACIENS
NEGATIVE
S. FAECALIS AND S. FAECALIS VAR. ZYMOGENES
HEMOLYSIS
POSITIVE
S. FAECALIS VAR. ZYMOGENES
NEGATIVE
S. FAECALIS
NEGATIVE
S. FAECIUM AND S. DURANS
FERMENTATION OF L-ARABINOSE
POSITIVE
S. FAECIUM
NEGATIVE
S. DURANS
STARCH HYDROLYSIS
POSITIVE
ATYPICAL S. FAECALIS (VEGETATION SOURCE)
NEGATIVE
PEPTONIZATION OF LITMUS MILK
POSITIVE
S. FAECALIS VAR LIQUEFACIENS (INSECT SOURCE)
NEGATIVE
ENTEROCOCCI (WARM-BLOODED ANIMAL SOURCES)
GROWTH AT 45 C ONLY
S. BOVIS AND S. EQUINUS
STARCH HYDROLYSIS
POSITIVE
LACTOSE FERMENTATION
ACID ONLY
S. BOVIS
NO CHANGE
S. EQUINUS
(LIVESTOCK AND POULTRY SOURCES)

Diagram 4. FLOW SHEET AND SEQUENCE OF TESTS TO PERFORM VERIFICATION STUDIES ON COLONIES BELIEVED TO BE FECAL STREPTOCOCCI

Example: Twenty-five sheen colonies on Endo-type membrane filter medium were subjected to verification studies shown in Diagram 1. Twenty-two of these colonies proved to be coliforms according to provisions of the test:

$$\text{Percent verification} = \frac{22}{25} \times 100$$

$$= 88$$

B A percent verification figure can be applied to a direct membrane filter count per 100 ml to determine the verified membrane filter count per 100 ml of the test organism.

Verified count per 100 ml of the test organism =

$$\frac{\text{Percent verification}}{100} \times \text{count per 100 ml of test organism}$$

Example: For a given sample, by a direct membrane filter test, the fecal coliform count was found to be 42,000 per 100 ml. Supplemental studies on selected colonies showed 92% verification.

$$\text{Verified fecal coliform count} = \frac{92}{100} \times 42{,}000$$

$$= 0.92 \times 42{,}000$$

$$= 38{,}640$$

Rounding off: = 39,000 per 100 ml

C A percentage of false-negative tests also can be determined (See II, C)

$$\text{Percent false negative} = \frac{\text{No. "negative" colonies found positive}}{\text{Total No. "negative" colonies tested}} \times 100$$

Example: On a total coliform test, 25 nonsheen (coliform negative) colony types were subjected to the coliform verification procedure shown in Diagram 1. Two of these colonies proved to be coliform colonies.

$$\text{Percent false negatives} = \frac{2}{25} \times 100$$
$$= 8$$

VI SOME APPLICATIONS OF PERCENT VERIFICATION CALCULATIONS

A In comparisons between two or more different membrane filter media, the medium which has the highest percentage of verification, and the lowest percentage of false negatives (based on a broad range of sample types and sources) is the better medium.

B In productivity comparisons between two or more different membrane filter media, the medium which produces the highest verified membrane filter counts per 100 ml (based on a broad range of sample types and sources) is the better medium.

C The worker is cautioned NOT to apply percentage of verification determined from one sample, to other samples. For example, do not determine a percentage verification on m-Endo broth for a sample taken from the Ohio River on September 6, and then seek to apply that percentage verification to another coliform determination from the Little Miami River, on the same date. Even the application of the verification percentage to another Ohio River sample, either on the same date from a different station, or on another date from the same station, should be undertaken with great caution. Such application of verification percentages from one sample to another should be taken only after sufficient studies have been made demonstrate the suitability of such a procedure.

VII USE OF VERIFICATION STUDIES IN MF-MPN COMPARISONS

A Comparisons of data obtained from MF versus MPN methods have been the source of great concern to microbiologists. For the current basis of comparisons, see Standard Methods (either 11th or 12th edition) "-- with a proviso that it should be used for determining the potability of drinking water only after parallel testing had shown that it afforded information equivalent to that given by the standard multiple-tube test."

B Some workers have sought to apply this requirement on the basis of statistical calculations, based on comparisons of numerical values from membrane filter tests with numerical values obtained from multiple-tube tests. Further study of this problem, and methods different workers have applied to the problem, can be made on the basis of the appended reference list

C Numerical comparisons between raw or verified membrane filter results on split samples, compared with multiple-tube results, also should take into account the question of the reliability of the multiple-tube test. The numerical results of the Completed Test for coliforms, for example, can be compared with the results of the Confirmed Test, to determine a percentage of verification for the multiple-tube test:

$$\text{Percent verification} = \frac{\text{Completed Test Coliforms per 100 ml}}{\text{Confirmed Test Coliforms per 100 ml}} \times 100$$

Example: On a given sample, the test was carried to the Completed Test stage. Afterward, both a Confirmed Test and a Completed Test coliform result were obtained, consisting of

Table 1. VALIDITY OF MF AND MPN "CONFIRMED TEST"*

Source	Number of supplies	MF Coliform Test			MPN Confirmed Test		
		Minimum	Maximum	Percent verified	Minimum	Maximum	Percent verified
Wells - Springs	16	1.0	7,600	96.6	7.0	11,000	64.6
Lakes - Lagoons	23	1.0	420,000	79.6	79	490,000	70.9
Creeks	19	32	260,000	75.8	120	460,000	66.4
Rivers	22	320	890,000	69.7	700	350,000	75.7
Sewage	11	1,400,000	28,000,000	68.6	460,000	49,000,000	73.8
Totals	91			78.1			70.3

*All coliform values are per 100 ml of sample

49,000 per 100 ml for the Confirmed Test and 33,000 per 100 ml for the Completed Test.

$$\text{Percent verification} = \frac{33,000}{49,000} \times 100$$

$$= 67$$

See Table 1 for some studies of MF verification studies, and parallel multiple-tube verification studies (Confirmed Test carried to Completed Test). These studies have been conducted in research laboratories of this Center, and demonstrate the difficulty and problems associated comparative evaluation of membrane filter versus multiple-tube methods. The student is invited to study this table at leisure.

REFERENCES

1 Delaney, J.E., McCarthy, J.A. and Grasso, R.J. Measurement of E. coli Type I by the Membrane Filter. Water and Sewage Works. 109, 289-294. 1962.

2 Geldreich, E.E., Clark, H.F., Huff, C.B. and Best, L.C. Fecal-Coliform-Organism Medium for the Membrane Filter Technique. JAWWA 57, 208-214. 1965.

3 Geldreich, E.E., Jeter, H.L. and Winter, J.A. Technical Considerations in Applying the Membrane Filter Procedures. In Press. 1966.

4 Hoffman, D.A., Kuhns, J.H., Stewart, R.C. and Crossley, E.I. A Comparison of Membrane Filter Counts and Most Probable Numbers of Coliform in San Diego's Sewage and Receiving Waters. JWPCF 36, 109-117. 1964.

5 McCarthy, J.A., Delaney, J.E. and Grasso, R.J. Measuring Coliforms in Water. Water and Sewage Works, 108, 238-243. 1961.

6 Thomas, H.A., Jr. and Woodward, R.L. Estimation of Coliform Density by the Membrane Filter and the Fermentation Tube Methods. American Journal Public Health 45, 1431-1437. 1955.

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Descriptors: Quality Control, Filters, Membrane, Enteric Bacteria, Microorganisms, Laboratory Tests

COLLECTION AND HANDLING OF SAMPLES FOR BACTERIOLOGICAL EXAMINATION

I INTRODUCTION

The first step in the examination of a water supply for bacteriological examination is careful collection and handling of samples. Information from bacteriological tests is useful in evaluating water purification, bacteriological potability, waste disposal, and industrial supply. Topics covered include: representative site selection, frequency, number, size of samples, satisfactory sample bottles, techniques of sampling, labeling, and transport.

II SELECTION OF SAMPLING LOCATIONS

The basis for locating sampling points is collection of representative samples.

A Take samples for potability testing from the distribution system through taps. Choose representative points covering the entire system. The tap itself should be clean and connected directly into the system. Avoid leaky faucets because of the danger of washing in extraneous bacteria. Wells with pumps may be considered similar to distribution systems.

B Grab samples from streams are frequently collected for control data or application of regulatory requirements. A grab sample can be taken in the stream near the surface.

C For intensive stream studies on source and extent of pollution, representative samples are taken by considering site, method and time of sampling. The sampling sites may be a compromise between physical limitations of the laboratory, detection of pollution peaks, and frequency of sample collection in certain types of surveys. First, decide how many samples are needed to be processed in a day. Second, decide whether to measure cycles of immediate pollution or more average pollution.

Sites for measuring cyclic pollution are immediately below the pollution source. Sampling is frequent, for example, every three hours.

A site designed to measure more average conditions is far enough downstream for a complete mixing of pollution and water. Keep in mind that averaging does not remove all variation but only minimizes sharp fluctuations. Downstream sites sampling may not need to be so frequent.

Samples may be collected 1/4, 1/2 and 3/4 of the stream width at each site or other distances, depending on survey objectives. Often only one sample in the channel of the stream is collected. Samples are usually taken near the surface.

D Samples from lakes or reservoirs are frequently collected at the drawoff and usually about the same depth and may be collected over this entire surface.

E Collect samples of bathing beach water at locations and times where the most bathers swim.

III NUMBER, FREQUENCY AND SIZE OF SAMPLES

A For determining sampling frequency for drinking water, consult the USEPA Standards.

1 The total number, frequency, and site are established by agreement with either state of USEPA authorities.

2 The minimum number depends upon the number of users. Figure 1 indicates that the smaller populations call for relatively more samples than larger ones. The numbers on the left of the graph refer to actual users and not the population shown by census.

3 In the event that coliform limits of the standard are exceeded, daily samples must be taken at the same site. Examinations should continue until two consecutive samples show coliform level is satisfactory. Such samples are to be considered as special samples and shall not be included in the total number of samples examined.

4 Sampling programs described above represent a minimum number which may be increased by reviewing authority.

B For stream investigations the type of study governs frequency of sampling.

C Collect swimming pool samples when use is heavy. The high chlorine level rapidly reduces the count when the pool is not in use. Residual chlorine tests are necessary to check neutralization of chlorine in the sample.

D Lake beaches may be sampled as required depending on the water uses.

Population served:	Minimum number of samples per month
25 to 1,000	1
1,001 to 2,500	2
2,501 to 3,300	3
3,301 to 4,100	4
4,101 to 4,900	5
4,901 to 5,800	6
5,801 to 6,700	7
6,701 to 7,600	8
7,601 to 8,500	9
8,501 to 9,400	10
9,401 to 10,300	11
10,301 to 11,100	12
11,101 to 12,000	13
12,001 to 12,900	14
12,901 to 13,700	15
13,701 to 14,600	16
14,601 to 15,500	17
15,501 to 16,300	18
16,301 to 17,200	19
17,201 to 18,100	20
18,101 to 18,900	21
18,901 to 19,800	22
19,801 to 20,700	23
20,701 to 21,500	24
21,501 to 22,300	25
22,301 to 23,200	26
23,201 to 24,000	27
24,001 to 24,900	28
24,901 to 25,000	29
25,001 to 28,000	30
28,001 to 33,000	35
33,001 to 37,000	40
37,001 to 41,000	45
41,001 to 46,000	50
46,001 to 50,000	55
50,001 to 54,000	60
54,001 to 59,000	65
59,001 to 64,000	70
64,001 to 70,000	75
70,001 to 76,000	80
76,001 to 83,000	85
83,001 to 90,000	90
90,001 to 96,000	95
96,001 to 111,000	100
111,001 to 130,000	110
130,001 to 160,000	120
160,001 to 190,000	130
190,001 to 220,000	140
220,001 to 250,000	150
250,001 to 290,000	160
290,001 to 320,000	170
320,001 to 360,000	180
360,001 to 410,000	190
410,001 to 450,000	200
450,001 to 500,000	210
500,001 to 550,000	220
550,001 to 600,000	230
600,001 to 720,000	240
720,001 to 780,000	250
780,001 to 840,000	260
840,001 to 910,000	270
910,001 to 970,000	280
970,001 to 1,050,000	290
1,050,001 to 1,140,000	300
1,140,001 to 1,230,000	310
1,230,001 to 1,320,000	320
1,320,001 to 1,420,000	330
1,420,001 to 1,520,000	340
1,520,001 to 1,630,000	350
1,630,001 to 1,730,000	360
1,730,001 to 1,850,000	370
1,850,001 to 1,970,000	380
1,970,001 to 2,060,000	390
2,060,001 to 2,270,000	400
2,270,001 to 2,510,000	410
2,510,001 to 2,750,000	420
2,750,001 to 3,020,000	430
3,020,001 to 3,320,000	440
3,320,001 to 3,620,000	450
3,620,001 to 3,960,000	460
3,960,001 to 4,310,000	470
4,310,001 to 4,690,000	480
4,690,001 or more	500

FIGURE I

E Salt water or estuarine beaches are sampled as needed with frequency depending on use.

F Size of samples depends upon examination anticipated. Generally 100 ml is the minimum size.

IV BOTTLES FOR WATER SAMPLES

A The sample bottles should have capacity for at least 100 ml of sample, plus an air space. The bottle and cap must be of bacteriological inert materials. Resistant glass or heat resistant plastic are acceptable. At the National Training Center, wide mouth ground-glass stoppered bottles (Figure 2) are used.

All bottles must be properly washed and sterilized. Protect the top of the bottles and cap from contamination by paper or metal foil hoods. Both glass and heat resistant plastic bottles may be sterilized in an autoclave. Hold plastic at 121°C for at least 10 minutes. Hot air sterilization. 2 hours at 170°C, may be used for dry glass bottles.

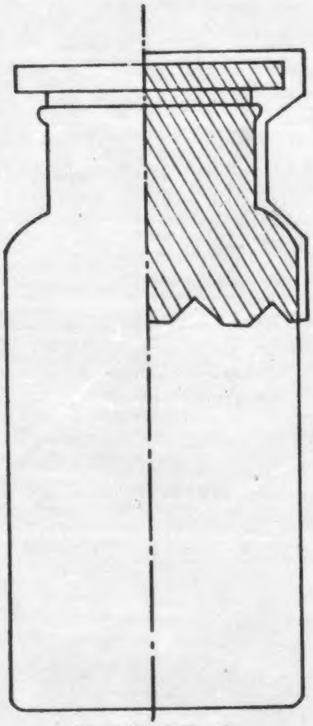


FIGURE 2

B Add sodium thiosulfate to bottles intended for halogenated water samples. A quantity of 0.1 ml of a 10% solution provides 100 mg per liter concentration in a 100 ml sample. This level shows no effect upon viability or growth.

C Supply catalogs list wide mouth ground glass stoppered bottles of borosilicate resistance glass, specially for water samples.

V TECHNIQUE OF SAMPLE COLLECTION

Follow aseptic technique as nearly as possible. Nothing but sample water must touch the inside of the bottle or cap. To avoid loss of sodium thiosulfate, fill the bottle directly and do not rinse. Always remember to leave an air space.

In sampling from a distribution system, first run the faucet wide open until the service line is cleared. A time of 2-3 minutes generally is sufficient. Reduce the flow and fill the sample bottle without splashing. Some authorities stress flaming the tap before collection, but the use of this technique is now generally considered as valueless. A chlorine determination is often made on the site.

B The bottle may be dipped into some waters by hand. Avoid introduction of bacteria from the human hand and from surface debris. Some suggestions follow: Hold the bottle near the base with one hand and with the other remove the hood and cap. Push the bottle rapidly into the water mouth down and tilt up up towards the current to fill. A depth of about 6 inches is satisfactory. When there is no current move the bottle through the water horizontally and away from the hand. Lift the bottle from the water, spill a small amount of sample to provide an air space, and return the uncontaminated cap.

C Samples may be dipped from swimming pools. Determine residual chlorine on the pool water at the site. Test the sample at the laboratory to check chlorine neutralization by the thiosulfate.

D Sample bathing beach water by wading out to the two foot depth and dipping the sample up from about 6 inches below the surface. Use the procedure described in V. B.

E Wells with pumps are similar to distribution systems. With a hand pumped well, waste water for about five minutes before taking the sample. Sample a well without a pump by lowering a sterile bottle attached to a weight. A device which opens the bottle underneath the water will avoid contamination by surface debris.

F Various types of sampling devices are available where the sample point is inaccessible or depth samples are desired. The general problem is to put a sample bottle in place, open it, close it, and return it to the surface. No bacteria but those in the sample must enter the bottle.

1 The J - Z sampler described by Zobell in 1941, was designed for deep sea sampling but is useful elsewhere (Figure) 3). It has a metal frame, breaking device for a glass tube, and sample bottle. The heavy metal messenger strikes the lever arm which breaks the glass tubing at a file mark. A bent rubber tube straightens and the water is drawn in several inches from the apparatus. Either glass or collapsible rubber bottles are sample containers.

Commercial adaptations are available.

2 Note the vane and lever mechanism on the New York State Conservation Department's sampler in Figure 4. When the apparatus is at proper depth the suspending line is given a sharp pull. Water inertia against the vane raises the stopper and water pours into the bottle. Sufficient sample is collected prior to the detachment of the stopper from the vane arm allowing a closure of the sample bottle.

The New York State Conservation Department's sampler is useful for shallow depths and requires nothing besides glass stoppered sample bottles.

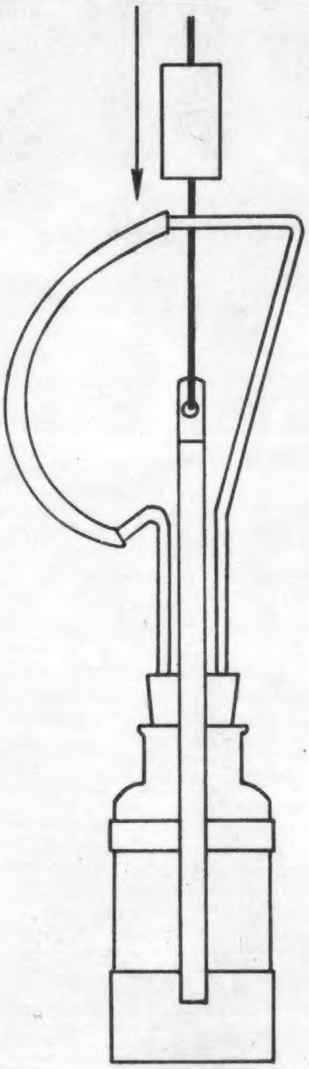


FIGURE 3

Reproduced with permission of the Journal of Marine Research 4:3, 173-188 (1941) by the Department of Health, Education and Welfare.

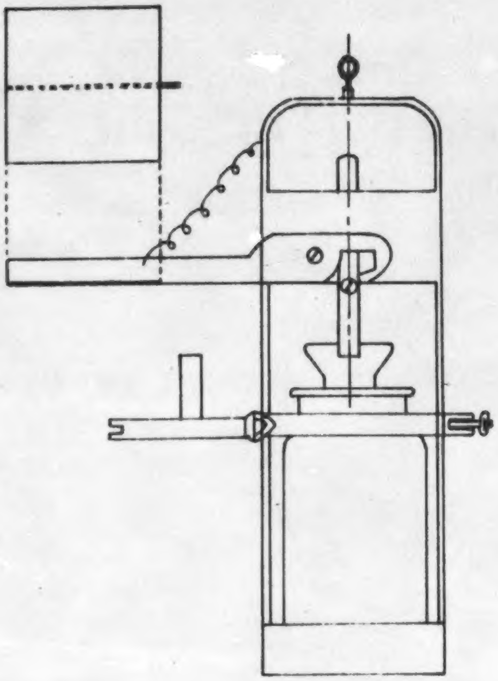


FIGURE 4

3 A commercial sampler is available which is an evacuated sealed tube with a capillary tip. When a lever on the support rack breaks the tip, the tube fills. Other samplers exist with a lever for pulling the stopper, while another uses an electromagnet.

VI DATA RECORDING

A Information generally includes: date, time of collection, temperature of water, locati of sampling point, and name of the sample collector. Codes are often used. The location description must be exact enough to guide another person to the site. Reference to bridges, roads, distance to the nearest town may help. Use of the surveyors' description and maps are recommended. Mark identification on the bottles or on securely fastened tags. Gummed tags may soak off and are inadvisable.

B While a sanitary survey is an indispensable part of the evaluation of a water supply, its discussion is not within the scope of this lecture. The sample collector could supply much information if desired.

VI SHIPPING CONDITIONS

The examination should commence as soon as possible, preferably within one hour. A maximum elapsed time between collection and examination is 30 hours for potable water samples and 6 hours for other water samples (time from collection to laboratory delivery). An additional 2 hours is allowed from delivery to laboratory to the completion of first-day laboratory procedures. Standard Methods (14th Edition) recommends icing of samples between collection and testing.

VII PHOTOGRAPHS

A photograph is a sample in that it is evidence representing water quality. Sample collectors and field engineers may carry cameras to record what they see. Pictures help the general public and legal courts to better understand laboratory data.

REFERENCES

1 APHA, AWWA, WPCF, Standard Methods for the Examination of Water and Wastewater. (12 Ed.) 1965.

2 Prescott, S.C., Winslow, C.E.A., and McCrady, M.H. Water Bacteriology. 6th Ed., 368 pp. John Wiley and Sons, Inc., New York. 1946.

3 Haney, P.D., and Schmidt, J. Representative Sampling and Analytical Methods in Stream Studies. Oxygen Relationships in Streams, Technical Report W58-2 pp. 133-42. U. S. Department of Health, Education and Welfare, Public Health Service, Robert A. Taft Sanitary Engineering Center, Cincinnati, Ohio. 1958.

4 Velz, C. J. Sampling for Effective Evaluation of Stream Pollution. Sewage and Industrial Wastes, 22:666-84. 1950.

5 Bathing Water Quality and Health III Coastal Water. 134 pp. U. S. Department of Health, Education, and Welfare, Public Health Service, Robert A. Taft Sanitary Engineering, Cincinnati, Ohio. 1961.

6 Zobell, C. E. Apparatus for Collecting Water Samples from Different Depths for Bacteriological Analysis. Journal of Marine Research, 4:3:173-88. 1941

This outline was originally prepared by A. G. Jose, former Microbiologist FWPCA Training Activities, SEC and updated by the Training Staff, National Training Center, DTTB, MDS, WPO, EPA, Cincinnati, OH 45268.

Descriptors: Equipment Microbiology, Sampling, Water Sampling

TESTING THE SUITABILITY OF DISTILLED WATER FOR THE BACTERIOLOGY LABORATORY

I INTRODUCTION

A Standard Methods for the Examination of Water and Wastewater (12th Edition) states;

"Only distilled water or demineralized water which has been tested and found free from traces of dissolved metals and bactericidal and inhibitory compounds may be used for the preparation of culture media and reagents. Bactericidal compounds may be measured by a biologic test procedure ...." This outline describes a suitable procedure.

B A need for such a test has been shown in the lack of reproducibility of plate counts and a possible cause of inconsistent results in split sample examinations.

II THEORY OF THE TEST PROCEDURE

A Growth of Aerobacter aerogenes in a chemically defined minimal growth medium. The addition of a toxic agent or a growth promoting substance will alter the 24 hr population by an increase or decrease of 20% or more, when compared to a control.

III APPARATUS AND MATERIALS

A Glassware - rinse all glassware in freshly redistilled water from a glass still. The sensitivity of the test depends upon the cleanliness of the sample containers, flasks, tubes, and pipettes. Use only borosilicate glassware.

B Culture - any strain of coliform IMViC type --++ (A. aerogenes). This can be easily obtained from any polluted river or sewage sample.

IV REAGENTS

A Use reagents of the highest purity. Some brands of potassium dihydrogen phosphate (KH_2PO_4) have large amounts of impurities. The sensitivity of the test is controlled in part by the purity of the reagents employed.

1 Carbon source - Sodium citrate, reagent, crystals ($Na_3C_6H_5O_7 \cdot 2H_2O$) 0.29 g dissolved in 500 ml of redistilled water.

2 Nitrogen source - Dissolve 0.60 g of ammonium sulfate, reagent, crystals, ($(NH_4)_2SO_4$) in 500 ml of redistilled water.

3 Salt mixture solution - Dissolve the following compounds in 500 ml of redistilled water.

Magnesium sulfate, reagent, crystals ($MgSO_4 \cdot 7H_2O$) 0.26 g.

Calcium chloride, reagent, crystals ($CaCl_2 \cdot 2H_2O$) 0.17 g.

Ferrous sulfate, reagent, crystals ($FeSO_4 \cdot H_2O$) 0.23 g.

Sodium chloride, reagent, crystals (NaCl) 2.50 g.

4 Phosphate buffer solution - Use a 1 to 25 dilution of a stock phosphate solution prepared by dissolving 34.0 gm of potassium dihydrogen phosphate (KH_2PO_4) in 500 ml of distilled water, adjusting to pH 7.2 with 1 N NaOH and diluting to 1 liter with distilled water.

5 Toxic control - dissolve 0.40 grams $CuSO_4 \cdot 5H_2O$ in 100 ml of redistilled water. Dilute 1:1000 for 1 mg per liter Cu before use.

B Sterilization of Reagents

Unknown distilled water sample - either boil for one minute or sterilize by membrane filtration.

Prepare reagents with redistilled water heated to boiling for 1 to 2 minutes. Phosphate buffer solution may be sterilized by MF filtration or boiling.

C Solutions are useful up to two weeks when stored at 5°C in sterilized glass stoppered bottles. The salts solution must be stored in the dark because sunlight results in copious ferric ion precipitation. A slight turbidity arising in the first 3 - 5 days does not detract from the usefulness of the reagents.

V PROCEDURE

A Collect 150 - 200 ml of water sample in a sterile borosilicate glass flask and sterilize. Label 3 flasks or tubes: A, B, and F. Add water Samples and redistilled water to each flask as indicated at the bottom of the page.

B Add a suspension of Aerobacter aerogenes (IMViC type --++) of such density that each flask will contain 25 - 75 cells per ml. Make an initial bacterial count by plating a 1 ml sample in plate count agar. Incubate tests A-F at 32° or 35°C for 20 - 24 hr. Make plate counts using dilutions of 1, 0.1, 0.01, 0.001 and 0.0001 ml.

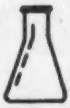
VI PREPARATION OF A BACTERIAL SUSPENSION

A Bacterial Growth

On the day prior to performing the distilled water suitability test, inoculate a strain of Aerobacter aerogenes onto a nutrient agar slant with a slope of approximately 2 - 1/2 inches in length contained in a 125 mm × 16 mm screw cap tube. Streak the entire agar surface to develop a continuous growth film and incubate 18 - 24 hrs at 35°C.

B Harvesting Viable Cells

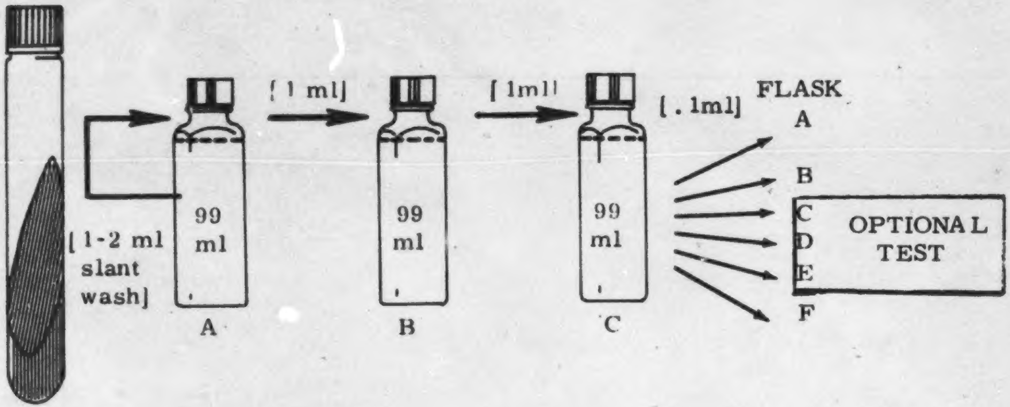
Pipette 1 - 2 ml of sterile dilution water from a 99 ml water blank onto the 18 - 24 hr culture. Emulsify the growth on the

	STANDARD TEST			OPTIONAL TEST		
	Control A	Unknown Dist. Water B	Toxic Control F	Food Available C	Nitrogen Source D	Carbon Source E
Media Reagents						
Citrate	2.5	2.5	2.5	X	2.5	X
Ammonium sulfate	2.5	2.5	2.5	X	X	2.5
Salt mixture	2.5	2.5	2.5	2.5	2.5	2.5
Phosphate buffer (7.3 ± .1)	1.5	1.5	1.5	1.5	1.5	1.5
Water, 1 mg per liter Cu	X	X	21.0	X	X	X
Unknown water	X	21.0	X	21.0	21.0	21.0
Redistilled water	21.0	X	X	5.0	2.5	2.5
TOTAL VOLUME	30.0	30.0	30.0	30.0	30.0	30.0

OPTIONAL TEST

OPTIONAL TEST

OPTIONAL TEST



slant by gently rubbing the bacterial film with the pipette, being careful not to tear the agar, and pour the contents back into the original 99 ml water blank.

C Dilution of Bacterial Suspension

Make a 1 - 100 dilution of the original bottle into a second water blank, and a further 1 - 100 dilution of the second bottle into a third water blank, shaking vigorously after each transfer. Then pipette 0.1 ml of the third dilution (1:1,000,000) into each of the flasks A, B, and F (see Standard Methods for Examination of Dairy Products, 12th ed.). This procedure should result in a final dilution of the organisms to a range of 25-75 viable cells for each ml of test solution.

D Verification of Bacterial Density

Variations among strains of the same organism, different organisms, media, and surface area of agar slopes will possibly necessitate adjustment of the dilution procedure to arrive at a specific density range between 25 - 75 viable cells. To establish the growth range numerically for a specific organism and medium, make a series of plate counts from the third dilution to determine the bacterial density. Then choose the proper volume from this third dilution which when diluted by the 30 ml in the flasks A, B, and F will contain 25 - 75 viable cells per ml. If the procedures are standardized as to surface area of the slant and laboratory technique, it is possible to reproduce results on repeated experiments with the same strain of microorganisms.

E Procedural Difficulties:

1 Chlorine or chloramine distilling over into receiver. Distilled water should be checked by a suitable quantitative procedure like the starch-iodide titration. If chlorine is found, sufficient sodium thiosulfate or sodium sulfite must be added.

2 Unknown water sample stored in soft glass containers or glass containers without liners for metal caps.

3 Contamination of reagents of distilled water with a bacterial background.

4 Incorrect dilution of A. aerogenes to get 25 - 75 cells per ml.

5 Gross contamination of the sample determined by the initial colony count before incubation.

F Calculation:

1 For growth inhibiting substances:

$$\frac{\text{colony count per ml Flask B}}{\text{colony count per ml Flask A}}$$

a Ratio 0.8 to 1.2 (inclusive) shows no toxic substances.

b Ratio less than 0.8 shows growth inhibiting substances in water sample.

2 For toxic control

$$\frac{\text{colony count per ml Flask F}}{\text{colony count per ml Flask A}} = \text{Ratio}$$

OPTIONAL TEST

3 *For nitrogen and carbon sources that promote growth**

$$\frac{\text{colony count per ml Flask C}}{\text{colony count per ml Flask A}} = \text{Ratio}$$

4 *For nitrogen sources that promote growth**

$$\frac{\text{colony count per ml Flask D}}{\text{colony count per ml Flask A}} = \text{Ratio}$$

5 *For carbon sources that promote bacterial growth**

$$\frac{\text{colony count per ml Flask E}}{\text{colony count per ml Flask A}} = \text{Ratio}$$

G Interpretation of Results:

1 The colony count from Flask A after 20 - 24 hours, at 35°C will depend on the number of organisms initially planted in Flask A and on the strain of *A. aerogenes* used in the test procedures. This is the reason the control Flask A must be run for each individual series of tests. However, for a given strain of *A. aerogenes* under identical environmental conditions, the terminal count should be reasonably constant when the initial plant is the same.

Thus, it is essential that the initial colony count on Flask A and Flask B should be approximately equal to secure accurate data.

2 When the ratio exceeds 1.2, it may be assumed that growth stimulating substances are present. However, this procedure is an extremely sensitive test and ratios up to 3.0 would have little significance in actual practice. Therefore, Test C, D, and E do not appear necessary except in special circumstances, when the ratio is between 1.2 and 3.0.

3 Usually Flask C will be very low and flasks D and E will have a ratio of less than 1.2 when the ratio of Flask B/ Flask A is between 0.8 and 1.2. The limiting factors of growth in Flask A are the nitrogen and organic carbon present. An extremely large amount of ammonia nitrogen with no organic carbon could increase the ratio in Flask D above 1.2 or the absence of nitrogen with high carbon concentration could give ratios above 1.2 in Flask E with an A/B ratio between 0.8 and 1.2.

4 A ratio below 0.8 indicates the water contains toxic substances and this ratio includes all allowable tolerances. As indicated in item 2 (above), the 1.2 ratio could go as high as 3.0 without any undesirable results.

5 We are unable to recommend corrective measures in specific cases of defective distillation apparatus. However, a careful inspection of the distillation equipment and a review of production and handling of the distilled water should enable the local laboratory personnel to correct the cause of the difficulty.

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*Do not attempt to calculate ratios, 3, 4, or 5 when ratio 1 indicates a toxic reaction.

**Ratio in excess of 1.2 indicates available source for bacterial growth.

CASE EXAMPLES

Test results for various distilled water samples

SOURCE	TEST COUNT	CONTROL COUNT	RATIO	INTERPRETATION
1	< 100	120,000	-----	Toxic Substance
2	74,000	170,000	0.4	Toxic Substance
3	18,000	14,000	1.3	Excellent water
4	21,000	14,000	1.5	Excellent water
5	310,000	60,000	5.2	Growth Substance
6	850,000	37,000	22.9	Growth Substance

REFERENCES

1 Standard Methods for Examination of Water and Wastewater. 12th Edition. 1965. p 578.

2 Geldreich, E. E. and H. F. Clark. Distilled Water Suitability for Microbiological Applications. Journal Milk and Food Technical. In Press. 1965.

This outline was prepared by E. E. Geldreich, Chief Bacteriologist, Water Supply Programs Division, WPO, EPA, Cincinnati, OH 45268.

Descriptors: Bacteria, Microbiology, Laboratory, Water Supply, Distillation, Water Quality Control

RESIDUAL CHLORINE AND TURBIDITY

I. INTRODUCTION

The Interim Primary Drinking Water Regulations (Federal Register, December 24, 1975) permits the options of substitution of up to 75 percent of the bacteriological samples with residual chlorine determinations. Any community or non-community water system may avail themselves of this option with approval from the State based upon results of sanitary surveys. Residual chlorine determinations must be carried out at the frequency of at least four for each substituted microbiological sample.

Since many potable water plants carry out their own microbiological determinations, it will be necessary that these laboratories be certified for the bacteriological parameters. Residual chlorine determinations may be carried out by any person acceptable to the State and the analytical method and techniques used must be evaluated in some manner to assure that reliable information is obtained.

Since the presence of high turbidity can interfere with the disinfection capability of chlorine, a maximum allowable limit has been set for turbidity as follows:

A. One turbidity unit (TU) as determined by a monthly average except that five or fewer turbidity units may be allowed if the supplier of water can demonstrate to the State that the higher turbidity does not

   1. Interfere with disinfection,
   2. Prevent maintenance of residual of disinfectant throughout distribution system, or,
   3. Interfere with microbiological determinations.

B. Five turbidity units based on an average of two consecutive days.

The Criteria and Procedures Document for Water Supply Laboratory Certification suggests that some quality control guidelines be instituted for the residual chlorine and turbidity measurements at the State level for the purpose of ensuring data validity for these critical measurements.

In response to public comments regarding the proposed Primary Regulations (Federal Register, December 24, 1975) it is stated that operators performing residual chlorine and turbidity analyses "....be certified, approved, or at least minimally trained to perform the analytical tasks before a State could accept their analytical determinations...."

II. RESIDUAL CHLORINE

Since residual chlorine analysis would be carried out in "field" conditions or in the small laboratories of treatment plants, perhaps by unskilled operators, it is necessary to keep the analytical method as simple as possible. For a number of years, operators had utilized the orthotolidine technique in a kit form to determine the chlorine residual. Recent studies and regulatory guidelines have dictated against this test procedure. The acceptable test procedure is now the DPD Test (13th Ed., Standard Methods for the Examination of Water and Wastewater, pgs. 129-132), for which kits are available from at least two companies and which meet requirements for accuracy and reliability. These kits are capable of measuring both free and combined chlorine of which only the free chlorine is measured to meet compliance requirements. Kit procedures call for a premeasured single powder or tablet reagent added to the test cell with the sample and a resultant color development measures by comparison the standardized colors within one minute. Standard Methods includes cautions regarding temperature and pH control regarding this test parameter and this test procedure, the DPD Test, is least effected by temperature and the pH is adjusted by the added reagents. The only interfering substance, oxidized manganese, can be determined in a preliminary step and compensated for in the final test value.

III. TURBIDITY

Turbidity has long been used in the water supply industry for indicating proper operational techniques. Turbidity should be clearly understood to be an expression of the optical property of a sample which causes light to be scattered and absorbed rather than transmitted in straight lines through the sample.

The standard method for the determination of turbidity has been based on the Jackson candle turbidimeter. However, the lowest turbidity value which can be measured directly on the Jackson turbidimeter is 25 units which is well above the monitoring level. Because of these low level requirements, the nephelometric method was chosen and procedures are given in Standard Methods (13th Ed., 1971).

IV. NEPHELOMETRIC MEASUREMENTS FOR COMPLIANCE MONITORING

The subjectivity and apparatus deficiencies involved in visual methods of measuring turbidity make each unsuitable as a standard method.

Since turbidity is an expression of the optical property of scattering or absorbing light, it was natural that optical instruments with photometers would be developed for this measurement.

The type of equipment specified for compliance monitoring(3,6) utilizes nephelometry.

A. Basic Principle(7)

The intensity of light scattered by the sample is compared (under defined conditions) with the intensity of light scattered by a standard reference solution (formazin). The greater the intensity of scattered light, the greater the turbidity. Readings are made and reported in NTUs (Nephelometric Turbidity Units).

B. Schematic

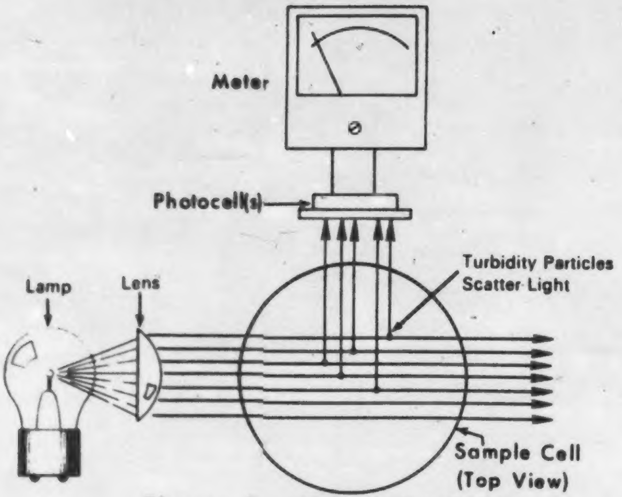

Figure 2 NEPHELOMETER
(90° Scatter)

Light passes through a polarizing lens and on to the sample in a cell. Suspended particles (turbidity) in the sample scatter the light.

Photocell(s) detect light scattered by the particles at a 90° angle to the path of the incident light. This light energy is converted to an electric signal for the meter to measure.

1. Direction of Entry of Incident Light to Cell

   a. The lamp might be positioned as shown in the schematic so the beam enters a sample horizontally.

   b. Another instrument design has the light beam entering the sample (in a flat-bottom cell) in a vertical direction with the photocell positioned accordingly at a 90° angle to the path of incident light.

2. Number of Photocells

   The schematic shows the photocell(s) at one 90° angle to the path of the incident light. An instrument might utilize more than one photocell position, with each final position being at a 90° angle to the sample liquid.

3. Meter Systems

   a. The meter might measure the signal from the scattered light intensity only.

   b. The meter might measure the signal from a ratio of the scattered light versus light transmitted directly through the sample to a photocell.

4. Meter Scales and Calibration

   a. The meter may already be calibrated in NTUs. In this case, at least one standard is run in each instrument range to be used in order to check the accuracy of the calibration scales.

   b. If a pre-calibrated scale is not supplied, a calibration curve is prepared for each range of the instrument by using appropriate dilutions of the standard turbidity suspension.

C. EPA Specifications for Instrument Design(7)

Even when the same suspension is used for calibration of different nephelometers, differences in physical design of the turbidimeters will cause differences in measured values for the turbidity of the same sample. To minimize such differences, the following design variables have been specified by the U. S. Environmental Protection Agency.

1. Defined Specifications

   a. Light Source

   Tungsten lamp operated at not less than 85% of rated voltage and at not more than rated voltage.

   b. Distance Traveled by Light

   The total of the distance traversed by the incident light plus scattered light within the sample tube should not exceed 10 cm.

   c. Angle of Light Acceptance of the Detector

   Detector centered at 90° to the incident light path and not to exceed ± 30° from 90°.

   (Ninety degree scatter is specified because the amount of scatter varies with size of particles at different scatter angles).

   d. Applicable Range

   The maximum turbidity to be measured is 40 units. Several ranges will be necessary to obtain adequate coverage. Use dilution for samples if their turbidity exceeds 40 units.

2. Other EPA Design Specifications

   a. Stray Light

   Minimal stray light should reach the photocell(s) in the absence of turbidity.

Some causes of stray light reaching the photocell(s) are:

1) Scratches or imperfections in glass cell windows.
2) Dirt, film or condensation on the glass.
3) Light leakages in the instrument system.

A schematic of these causes is shown in Figure 3.

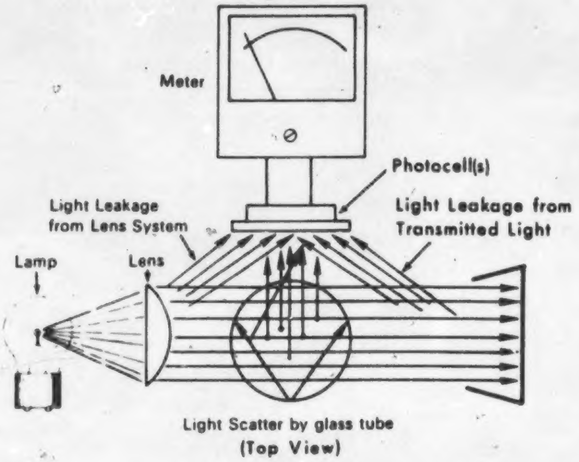
Meter
Photocell(s)
Light Leakage from Lens System
Light Leakage from Transmitted Light
Lamp
Lens
Light Scatter by glass tube
(Top View)

Figure 3 NEPHELOMETER SOURCES OF STRAY LIGHT

Stray light error can be as much as 0.5 NTU. Remedies are close inspection of sample cells for imperfections and dirt, and good design which can minimize the effect of stray light by controlling the angle at which it reaches the sample.

b. Drift

The turbidimeter should be free from significant drift after a short warm-up period. This is imperative if the analyst is relying on a manufacturer's solid scattering standard for setting overall instrument sensitivity for all ranges.

c. Sensitivity

In waters having turbidities less than one unit, the instrument should detect turbidity differences of 0.02 unit or less. Several ranges will be necessary to obtain sufficient sensitivity for low turbidities.

3. Examples of instruments meeting the specifications listed in 1 and 2 above include:

a. Hach Turbidimeter Model 2100 and 2100A.

b. Hydroflow Instruments DRT 100, 200, and 1000.

4. Other turbidimeters meeting the listed specifications are also acceptable.

D. Sources of Error

1. Sample Cells

   a. Discard scratched or etched cells.

   b. Do not touch cells where light strikes them in instrument.

   c. Keep cells scrupulously clean, inside and out.(8)

      1) Use detergent solution.

      2) Organic solvents may also be used.

      3) Use deionized water rinses.

      4) Rinse and dry with alcohol or acetone.

2. Standardizing Suspensions(7)

   a. Use turbidity - free water for preparations. Filter distilled water through a 0.45μm pore size membrane filter if such filtered water shows a lower turbidity than the distilled water.

   b. Prepare a new stock suspension of Formazin each month.

   c. Prepare a new standard suspension and dilutions of Formazin each week.

3. Sample Interferences

   a. Positive

      1) Finely divided air bubbles

   b. Negative

      1) Floating debris

      2) Coarse sediments (settle)

      3) Colored dissolved substances (absorb light)

E. Reporting Results(7)

NTU	RECORD TO NEAREST
0.0-1.0	0.05
1-10	0.1
10-40	1
40-100	5
100-400	10
400-1000	50
>1000	100

F. Precision and Accuracy(7)

1. In a single laboratory (EMSL), using surface water samples at levels of 26, 41, 75 and 180 NTU, the standard diviations were ±0.60, ±0.94, ±1.2 and ±4.7 units, respectively.

2. Accuracy data is not available at this time.

V. STANDARD SUSPENSIONS AND RELATED UNITS(9)

One of the critical problems in measuring turbidity has been to find a material which can be made into a reproducible suspension with uniform sized particles. Various materials have been used.

A. Natural Materials

1. Diatomaceous earth
2. Fuller's earth
3. Kaolin
4. Naturally turbid waters.

Such suspensions are not suitable as reproducible standards because there is no way to control the size of the suspended particles.

B. Other materials

1. Ground glass
2. Microorganisms
3. Barium Sulfate
4. Lates spheres

Suspensions of these also proved inadequate.

C. Formazin

1. A polymer formed by reacting hydrazine sulfate and hexamethylenetetramine sulfate.

2. It is more reproducible than previously used standards. Accuracy of ± one percent for replicate solutions has been reported.

3. In 1958, the Association of Analytical Chemists initiated a standardized system of turbidity measurements for the brewing industry by:

   a. Defining a standard formula for making stock Formazin solutions and

   b. Designating a unit of measurement based on Formazin, i.e., the Formazin Turbidity Unit (FTU).

4. During the 1960's Formazin was increasingly used for water quality turbidity testing. It is the currently recognized standard for compliance turbidity measurements.

D. Units

1. At first results were translated into Jackson Turbidity Units (JTU). However, the JTU was derived from a visual measurement using concentrations (mg/liter) of silica suspensions prepared by Jackson. They have no direct relationship to the intensity of light scattered at 90 degrees in a nephelometer.

2. For a few years, results of nephelometric measurements using specified Formazin standards were reported directly as Turbidity Units (TUs).

3. Currently, the unit used is named according to the instrument used for measuring turbidity. Specified Formazin standards are used to calibrate the instrument and results are reported as Nephelometric Turbidity Units (NTUs).

VI. SUMMARY

The importance of residual chlorine determination can be seen in its possible effect on the health of the consumers. The Criteria and Procedures for Laboratory Certification suggests that some form of quality assurance should be instituted on a state level to assure valid data for both tne chlorine and turbidity measurements. The comments on the public responses to the proposed Interim Primary Regulations also suggests some form of quality assurance on the state level to be instituted. Consequently, the Regional Certification team should point out to the principal laboratories the importance of some kind of effort being instituted. States might wish to offer some kind of formal training effort as part of the approval mechanism for the operators doing the chlorine and/or turbidity measurements.